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Neuroendocrine Control of Maternal Behaviour

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Declaration

I declare that this thesis is entirely my own composition and that the work is my own unless clearly indicated as done in collaboration where I contributed substantially to the work. I also declare that the work completed has not been submitted for any other degree or professional qualification by myself.

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To Vickie, Liv and Olivia, whose ears were always available to bend on a bad day but were also the first to cheer when it did work and for those unforgettable trips to Amsterdam and Norway. Matt, for your 'faith' in me and oh, you can come back from the hills now and take me out for that celebratory beer. Mum, for being the intermediate between Dad and I and your continuing encouragement. Dad, you can stop the nagging now, it's done!

Abstract

Maternal behaviour during the peri-partum period, albeit in differing forms, can be observed in all mammals, thus it must serve an important evolutionary purpose in enabling the successful raising of offspring. Maternal behaviour is comprised of a large suite of behaviours; in rodents these are generally defined as lactation, pup retrieval, maternal aggression and pup grooming. The maternal behaviour circuitry involves many brain regions including the hypothalamus and the limbic system which work together to regulate the motor, motivational and emotional demands of the lactation period. The main aim of this thesis is to understand how different neuromodulators, specifically oxytocin (OXT), vasopressin (AVP), allopregnanolone (AP) and GABA, influence the expression of maternal behaviour, especially maternal aggression, and where in the brain they act to control this.

Maternal aggression in rats changes dramatically throughout pregnancy, parturition and lactation. This expression is highly influenced by pups and during early lactation, pup cues are essential in maintaining it. Towards the end of lactation pup cues appear to result in the down regulation of maternal aggression. The maternal aggression circuitry is highly complex and involves many of the brain regions highlighted to be involved in maternal behaviour. The neuropeptides, OXT and AVP, are observed to have significant changes in their systems that correlate with maternal aggression, specifically within the BnST and PVN. This leads to the proposal they work oppositely to control maternal aggression by regulating fear and anxiety in the lactating rat. There is also evidence the OXT system mediates the motor output of maternal aggression. AP and GABA are also important in maternal behaviour, especially in relation to fear; whether this in context with OXT to enable maternal aggression or if they are a back up mechanism for OXT secretion malfunctioning remains to be determined.

By understanding the complex maternal behaviour neural circuitry and how neuromodulators work to control it, enables the development of potential therapies for disorders a woman may experience during the peri-partum period. Prevention of these disorders is not only beneficial to the mother and her immediate family but is also crucial for her offspring's development in prevention of adulthood disorders stemming from their childhood experience which can impact their own paternal or maternal care ability.

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Abbreviations

5α-DHP	=	5α-dihydroprogesterone
3α, 5α-THP	=	3α, 5α-tetrahydroprogesterone (or AP)
AAF	=	Acetic alcohol fixative
ABC	=	Avidin-biotin complex
AH	=	Anterior hypothalamus
AHN	=	anterior hypothalamic nucleus
AMY	=	Amygdala (whole)
ANOVA	=	Analysis of variance
AP	=	Allopregnanolone
AVP	=	Vasopressin
AVP-R	=	Vasopressin receptor
BnST	=	Bed nucleus of stria terminalis
BnSTpr	=	principal BnST
BM	=	Bicuculline Methiodide
BOBX	=	Olfactory bulb removal
CeA	=	Central amygdala
CRH	=	Corticotrophin releasing hormone
DAB	=	Diaminobenzidine tetrachloride
DHEAS	=	Dehydroepiandrosterone sulphate
DNA	=	Deoxyribonucleic acid
EPM	=	Elevated plus maze
FIN	=	Finasteride
GABA	=	γ-aminobutyric acid
GAD	=	Glutamic acid decarboxylase or glutamate decarboxylase
HAB	=	High anxiety behaviour
HPA	=	Hypothalamic-Pituitary-Adrenal
h	=	Hour
ICC	=	Immunocytochemistry
ICV	=	Intracerebroventricular
IEG	=	Immediate early gene
Ig	=	Immunoglobulin
ISH	=	In situ hybridisation
i.p.	=	Intraperitoneal
KO	=	Knock out
LAB	=	Low anxiety behaviour
LD	=	Lactation day
LS	=	Lateral septum
LSv	=	ventral LS
MeA	=	Medial amygdala
MPOA	=	Medial preoptic area
min	=	Minute
mRNA	=	Messenger ribonucleic acid
NAccs	=	Nucleus accumbens
OB	=	Olfactory bulbs
OTR	=	Oxytocin receptor
OXT	=	Oxytocin

PAG	=	Periaqueductal grey area
PB-T	=	PB with 0.2% Triton X-100
PD	=	Day of parturition
PDnumber	=	Pregnancy day number
PMd	=	dorsal premammillary nucleus
PMT	=	Premenstrual tension
PVN	=	Paraventricular nucleus
SON	=	Supraoptic nucleus
s.c.	=	Subcutaneous
RIA	=	Radioimmunoassay
RNA	=	Ribonucleic acid
THIP	=	4,5,6,7-tetrahydroisoxazolo-(5,4-c-)-pyrindin-5-ol
VMNdm,c	=	Dorsomedial and central areas of the ventromedial hypothalamic nucleus

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Chapter One: General Introduction

1.1 Maternal Behaviour

Maternal behaviour can be observed in all vertebrates from birds to rodents to humans. Although maternal behaviour expression varies greatly between species, it's main objective to ensure offspring survival and hence that of the species remains the same. Maternal behaviour consists of a whole suite of behavioural components displayed during the peri-partum period which enable the successful raising of offspring; in rodents these are generally defined as [1]:

- Pup retrieval – the process of picking up a pup in the mouth and moving it to the nest area.
- Nursing – feeding pups by their attachment to the dam's nipple and suckling to induce milk ejection.
- Nest building – constructing a nest using suitable material (e.g. paper and wood shavings) that is used for birth and looking after pups.
- Pup grooming and licking – general cleaning of the pups.
- Maternal aggression – exhibition of offensive and defensive behaviour in defence of nest and pups from harmful con- or inter-specific intruder

Each maternal behaviour component has distinct expression throughout the peri-partum period often closely related to the physical and behavioural changes in the young as they develop (see appendix 1 for a brief description of changes to specific maternal behaviour components during lactation) [1, 2].

Maternal behaviour expression is greatest immediately after parturition but its display can be induced during late pregnancy in all primiparous and multiparous rats around 3.5h before parturition coinciding with the onset of vigorous uterine

contractions [1, 3, 4]. These experiments imply that parturition is a critical time point when the physiological processes of pregnancy that prime the female for maternal behaviour become receptive to external cues to enable expression and maintenance of maternal behaviour i.e. sensory information at parturition stimulates an increase in maternal responsiveness [1, 5]. If the pelvic nerve is cut in hormonally primed pregnancy-terminated rats, they display a prolonged latency to express maternal responsiveness compared to intact sham controls leading to the hypothesis that pelvic nerve excitation (as occurs during parturition) enhances maternal responsiveness [5]. Oxytocin (OXT) secretion is indicated as the mechanism linking an increase in maternal responsiveness to pelvic nerve excitation because stimulation of the vagina and cervix is known to result in pituitary OXT release (the role of OXT in maternal behaviour onset is discussed further in section *1.4*) [5].

Maternal behaviour develops around parturition in all primiparous rats and mice with no differences observed compared to multiparous rats [6-8]. If the pups are immediately removed after birth then little maternal behaviour is expressed by the lactating rat in a subsequent maternal behaviour test with pups [1, 2]. However, maternal behaviour is expressed in virgin female rats but only after exposure to pups continuously for at least 7 days; a process known as pup-sensitization [4, 9, 10]. Thus, it may be proposed that the brain has evolved a mechanism to switch maternal behaviour on and off allowing the appropriate behaviour to be exhibited only at the correct life history stage. Rosenblatt and Lehrman (1963) therefore proposed that pregnancy ‘primes’ the rat ready for the onset of maternal behaviour but post-partum stimulation from pups is essential for full maternal behaviour development [1, 11, 12]. The influence of these two mechanisms, the hormonal change of pregnancy and

parturition and pup cues, on maternal behaviour will be discussed in the next sections of this chapter.

It is essential to understand maternal behaviour because the maternal care a pup or young receives during their formative time can have lifelong behavioural and physiological consequences [13-22]. For example, the type of maternal separation (a stressor for pups) a female pup experiences during the post-partum period can programme their physiology, behaviour and anxiety levels which then impacts upon the maternal care they display as adults [15]. Brief daily maternal separation (15 min), when a pup, causes lower acute stress-induced anxiety behaviour and hypothalamic-pituitary-adrenal (HPA) axis activation during adulthood for a female rat, whereas long maternal separation (3h) result in higher levels [15]. Furthermore, female rats who experienced brief maternal separation as a pup exhibit greater maternal aggression and better maternal care than dams who experienced long periods maternal separation [15]. Maternal separation in both male and female mice when pups results in higher anxiety behaviour on the elevated plus maze (EPM), open-field and novel object exploration tests in adulthood [16]. Post natally stressed dams display poor maternal care which for male rat offspring results in heightened anxiety and aggressive behaviour during adulthood [23-25].

It is not only the experience during the postnatal time period that is crucial in programming adult behaviour, but experiences during the prenatal period also have consequences. Prenatal stress for dams significantly increases anxiety behaviour in female offspring [17]. In adulthood, the female offspring of prenatally stressed dams display poorer maternal care during lactation themselves indicating prenatal stress in dams causes long-lasting behavioural changes in offspring. Prenatal stress also altered

the HPA axis as an increase in HPA axis activity is observed in the female offspring of prenatally stressed dams as compared to controls and virgins to a mild stressor [17]. Thus, pre- and post-natal experiences can programme HPA reactivity to stressors which can have lifelong consequences causing mood or behavioural disorders in offspring during adulthood; therefore it is important to understand how maternal behaviour is regulated to enable prevention and possible treatment of these disorders [14, 17].

1.1.1 Non-hormonal influences on maternal behaviour

There are two important features in the control of maternal behaviour highlighted by research into pup-sensitized virgin rats. The first is that the hormonal experience of pregnancy is essential for the full expression of maternal behaviour. Virgin rats will not exhibit maternal behaviour unless sensitized to pups continuously for at least 7 days [10]. Pup-sensitization was concluded not to be due to changing circulating ovarian hormones levels as no effect of oestrous cycle stage or ovariectomy was observed on pup-sensitization latency [10]. However, when the intensity of maternal behaviour displayed was compared between intact and ovariectomised pup-sensitized virgin rats, it was observed that intact rats retrieved pups faster, built better nests and spent more time in the nest than ovariectomised rats [26, 27]. This suggests that the endocrine background of the ovarian hormone cycling rat enables improved maternal behaviour quality once maternal behaviour has been stimulated by pups [26, 27]. The long latency until virgins express maternal behaviour is proposed to be due to the fear of pups virgin female rats normally exhibit and that they therefore must become habituated to the pups before they can express maternal behaviour [3, 28, 29]. For example, if virgin female rats are sensitized to

pups in a large cage compared to small, the latency to express maternal behaviour is greater as the rats are able to avoid pups and do not need to overcome their aversion [4].

One of the main negative stimuli from the pups must be olfactory cues because anosmia (lack of olfactory functioning) in virgin female rats reduces the latency to express maternal behaviour [4, 30]. In pregnant rats, it is assumed that the hormonal influences during pregnancy and at parturition reduce fear to enable her to express maternal behaviour immediately after birth (see section 1.1.2) [3]. Fear and anxiety are observed to decrease throughout pregnancy and into lactation with evidence from studies of behaviour on the EPM, in open field paradigms and from HPA axis responses [3, 29, 31-40]. Pup-sensitized virgin rats also cannot exhibit a full lactating posture because they do not experience the hormonal changes which induce milk production and nipple development so pups cannot attach to feed [4, 41].

The second is that once maternal behaviour is initiated regardless of prior hormonal experience pup stimulation is essential for maternal behaviour maintenance and re-establishment [4]. Latency to retrieve pups is similar to lactating rat levels once maternal behaviour is initiated in pup-sensitized virgin rats [2, 4, 41, 42]. No differences are observed in nest building or time spent in the nest between pup-sensitized virgin female and lactating rats [4]. Previous maternal experience, irrespective of whether it had been lactation or pup-sensitization, was reported to reduce latency to express maternal behaviour when rats were presented with foster pups outside of the lactation period [4, 27]. This indicates that maternal responsiveness is rapidly switched on at the time of parturition, which is probably

controlled by hormonal factors, but once initiated maternal behaviour is maintained by pup cues [43].

Pup-sensitized ovariectomised virgin rats provide a baseline of maternal behaviour expression under non-hormonal conditions with which the efficacy of exogenous hormonal applications in inducing maternal behaviour can be compared; this provides a useful model to try to understand the complexity of maternal behaviour regulation [44].

1.1.2 Hormonal influences on maternal behaviour

As pregnancy proceeds dramatic changes occur in the ovarian hormones (estrogen and progesterone) and the pituitary hormone, prolactin (Fig 1.1); it is these hormonal changes that are proposed to develop the ability for dams to express maternal behaviour [3]. Ovariectomy in late pregnant rats reduces the likelihood of maternal behaviour display by 50% indicating that ovarian hormones are important in the initiation of maternal behaviour [45]. In non-pregnant ovariectomised rats, treatment with estrogen, progesterone and prolactin to resemble their fluctuations during pregnancy is able to significantly reduce the latency for maternal behaviour onset from 7 days when no hormonal manipulation to 35-40h [46]. Treatment with only estrogen and progesterone was also able to reduce maternal behaviour onset latency but not to the extent when all three hormones were administered together [46]. This was hypothesised to be due to stimulation of prolactin release by estrogen from the anterior pituitary [46-49]. Evidence of this hypothesis is that estrogen and progesterone implants releasing physiological amounts over a specific time (i.e. to mimic pregnancy) significantly reduced maternal behaviour onset latency [27, 50]. The same effect was observed even if the progesterone implant was removed prior to

estrogen treatment [27, 50]. Hence in both instances, application of exogenous progesterone and estrogen alone was able to reduce maternal behaviour onset latency without the need for exogenous prolactin.

From Fig. 1.1, it can be observed that circulating progesterone levels rapidly decline during late pregnancy whilst estrogen increases, so there is a rapid change in the estrogen to progesterone ratio. Thus it was proposed that progesterone withdrawal 'primes' the pregnant rat to become sensitive to the estrogen rise which enables maternal behaviour onset [3, 46]. If hysterectomised, ovariectomised pregnancy-terminated rats are treated with estrogen at the time of surgery and later progesterone, rapid development of maternal behaviour occurred similar to hysterectomised pregnancy- terminated rats [44, 51]. (Hysterectomy causes a similar change in ratio of progesterone to estrogen as observed at late pregnancy). Furthermore, it was shown that treatment with estrogen alone in these hysterectomised, ovariectomised pregnancy-terminated rats was effective in reducing latency for maternal behaviour onset but the amount of estrogen required was supraphysiological [44, 51]. Supraphysiological estrogen applications alone are also able to induce maternal behaviour in ovariectomised virgin rats whereas normal physiological doses could not elicit maternal behaviour [27, 46]. Physiological estrogen levels only induced maternal behaviour in hysterectomised and ovariectomised virgin rats if they had prior progesterone exposure and withdrawal, this is evidence that progesterone withdrawal reduces the estrogen threshold allowing normal pregnancy levels to facilitate maternal behaviour [27]. Withdrawal of progesterone is essential as maintaining high progesterone levels along with estrogen in an ovariectomised virgin rat inhibited maternal behaviour onset [52]. Injecting progesterone to prevent its

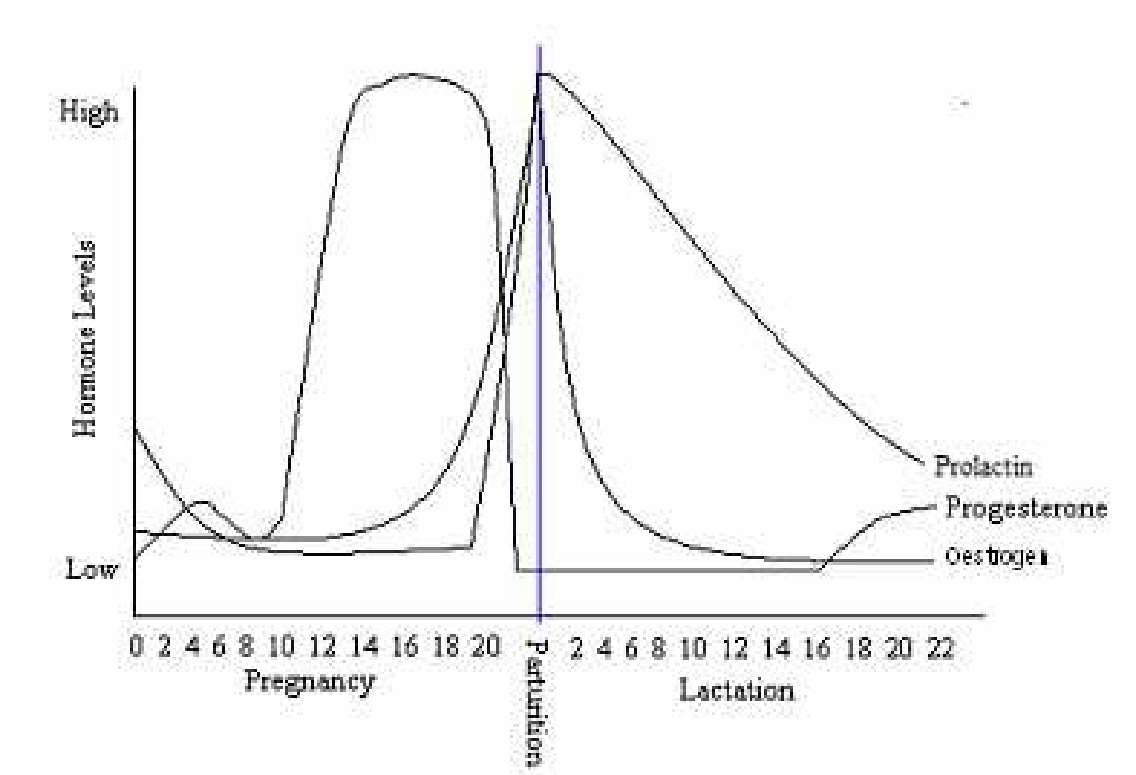


Figure 1.1: Circulating hormone levels during pregnancy, parturition and lactation in rats. Adapted from Gammie and Lonstein (2006) and Rosenblatt *et al* (1998) [53, 54].

normal decline inhibited the rapid induction of maternal behaviour in hysterectomised only or hysterectomised, ovariectomised pregnancy-terminated rats treated with estrogen [27]. Administration of progesterone at the same time as supraphysiological estrogen levels to hysterectomised, ovariectomised virgin rats also inhibited maternal behaviour induction [27, 44].

The change in the estrogen and progesterone ratio occurs at the same time point as maternal behaviour is first observed, just prior to the beginning of delivery (see results in chapter 4 of this study), and hence it was proposed to be the point of maternal behaviour initiation [3]. Hysterectomy to terminate pregnancy in rats was observed to cause an increase in maternal responsiveness [3, 44]. Ovariectomy at the same time as hysterectomy in pregnant rats increased the maternal responsiveness onset latency providing more evidence that these ovarian hormones are necessary for increased maternal responsiveness expression [3, 55]. At the end of pseudopregnancy

(when the animal displays signs of pregnancy along with similar hormonal changes, following mating with a sterile male, but is not actually pregnant), changes in circulating estrogen and progesterone levels resemble those of late pregnancy and during this time pseudopregnant rats express increased maternal responsiveness [3]. Estrogen levels start to rise around pregnancy day 15 and it is this early rise that is important for maternal behaviour initiation because ovariectomy performed on pregnancy day 20 or 21 does not prevent maternal behaviour development [51].

This is not the complete story because although maternal behaviour was able to be induced in ovariectomised nulliparous rats with just estrogen and progesterone administration, it was a combination of estrogen, progesterone and prolactin which resulted in the shortest maternal behaviour onset latency [46, 50]. Also, hypophysectomised (to remove the source of prolactin) rats treated with estrogen/progesterone failed to reduce latencies to become maternal unlike estrogen/progesterone treatment alone indicating circulating pituitary hormones, such as prolactin, may be involved in maternal behaviour onset [56]. Prolactin is essential in maternal behaviour initiation because administration of bromocriptine, a dopamine agonist which prevents the release of prolactin, to estrogen and progesterone treated ovariectomised nulliparous rats blocked the rapid onset of maternal behaviour [57]. This block in rapid maternal behaviour onset also occurred when rats were hypophysectomised and in both cases this block could be prevented by application of prolactin [57-59]. This research supported the theory mentioned above that estrogen administration acts to enhance prolactin release, thus enabling reduced maternal behaviour latencies when only estrogen and progesterone were applied to the ovariectomised nulliparous rat [48-50]. The effect of prolactin on the initiation of

maternal behaviour was demonstrated to be dependent on having had prior experience of these ovarian hormones as prolactin administration alone in the nulliparous rat did not reduce maternal behaviour onset latency [57]. Furthermore, it was shown that a regimen of both estrogen and progesterone treatment was required, as giving either separately with prolactin did not result in the rapid onset of maternal behaviour [57, 60].

Central prolactin effects are crucial for maternal behaviour initiation because both ICV and direct medial preoptic area (MPOA) infusions of prolactin reduced maternal behaviour onset latency whereas systemic injections had no effect [57, 60, 61]. This effect on the initiation of maternal behaviour was not restricted to ovine prolactin as it also could be induced by human placental lactogen, rat prolactin and rat placental lactogens (I and II) [60-62]. Therefore the central actions of any lactogenic hormones maybe crucial for the onset of maternal behaviour, with recent research indicating that it is their interactions with the prolactin receptor are essential [60-62]. Mice with a null mutation for the prolactin receptor (allowing examination of behaviour without manipulating any lactogenic hormone level) exhibit a maternal behaviour deficit phenotype, especially in pup retrieval [63]. This was specific to maternal behaviour as no deficiency was observed in the water-maze, a spatial (cognitive) memory test [63]. In addition, ICV application of a specific prolactin receptor antagonist, S179D-PRL, blocked the rapid onset of maternal behaviour in estrogen, progesterone primed, nulliparous rats [64]. This remained the case when applied to the MPOA (a brain region significantly linked with maternal behaviour, see section 1.1.3) where dramatic changes in prolactin receptor immunoreactivity and mRNA expression are observed during the peri-partum period [64-66]. Hence the full

experience of the hormonal changes during pregnancy are required for the display of full maternal behaviour where rising levels of progesterone followed by its natural decline act in the brain to prime the rat to respond fully to rising estrogen and prolactin levels to initiate maternal behaviour.

1.1.3 Maternal behaviour: brain circuitry

Although the exhibition of maternal behaviour components may occur alongside each other (for example pup licking may occur whilst the dam is nursing) usually the brain is required to rapidly switch the expression from one maternal behaviour to another [67]. As each maternal behaviour results in its own specific motor output, it must therefore have its own unique neuronal circuitry [67]. However, to ensure each behaviour is expressed appropriately, there must be some commonalities in these neural circuits which control the dynamic switch in maternal behaviour expression [67]. The brain must therefore have complex neural circuitry regulating maternal behaviour and research, using mainly lesion and immunocytochemistry (ICC) techniques, are elucidating brain regions involved and which aspects of maternal behaviour they feature. Other techniques used include in-situ hybridisation (ISH), histochemical tracers and clinical brain imaging such as magnetic resonance imaging (MRI) and these may be mentioned as appropriate.

Brain lesions destroy neurones and are generally performed in one particular region of the brain preventing the flow of information from that region to another; hence indicate that that brain region is required for the control of a specific behaviour. They can be performed in numerous ways including knife cuts, excitotoxic or electrical methods. Electrical lesions destroy both neurones and fibre projections of

the brain region whereas excitotoxic lesions just destroy specific neuron cell bodies [27].

Another way researchers examine the maternal behaviour circuitry is to employ immediate early gene (IEG) ICC. The IEGs normally investigated include Fos, pCREB, Fos B and Egr-1 (also known as Zif). IEGs generate proteins which act as transcription factors and therefore link extracellular input signalling with genomic outputs. For example Fos can bind to the activator protein-1 site, which is on the promoter region of many genes, to induce their transcription [68-71]. A change in the electrical activity of a cell causes an immediate up regulation of IEGs [12]. Thus the ability to examine IEG expression within the rodent brain provides indirect evidence of neural activity in specific brain areas during exhibition of a behaviour of interest [12]. However it is important to note that IEGs only provide information about postsynaptic activity and cannot tell us about the nature of the synaptic input to these neurones [28]. Furthermore they only reflect activation in the brain region during the behaviour display of behaviour and provide no evidence of how it is regulating the behaviour [12, 28].

The IEG, Fos, can be used in two main ways to investigate the maternal brain circuitry; the first is to observe if altered behavioural expression results from changes in functional subcellular mechanisms induced by Fos [70]. The second, and the method used in this thesis, is to map Fos expression in specific brain regions in rodents following exposure to different experimental conditions as an indicator of whether the neuron have been stimulated [70].

The maternal brain circuitry is complex and involves many different brain regions highlighted by lesion or IEG studies (see Fig. 1.2). The next sections of this

chapter will examine these brain regions and discuss how the lesion and IEG studies provide evidence of how they are important and what their function may be in the regulation of maternal behaviour.

1.1.3.1 Medial preoptic area and bed nucleus of stria terminalis

The MPOA is a brain region described with varying roles in females and males, i.e. it is sexually dimorphic in nature. In male rats, only, the MPOA is essential for sexual behaviour [72-75]. In females, the MPOA is critical for maternal behaviour (although

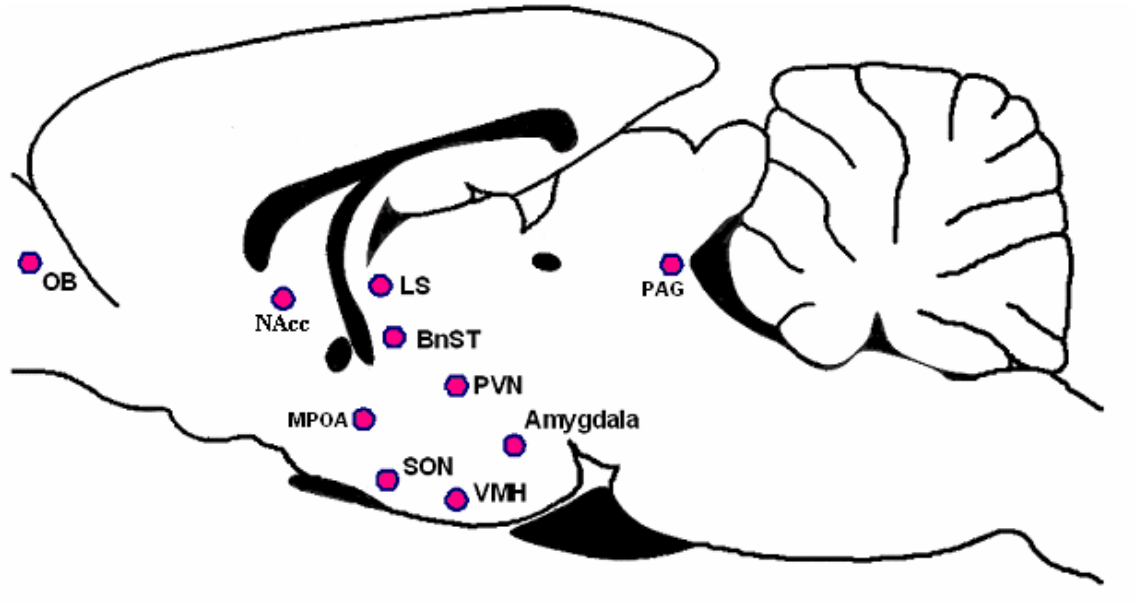


Figure 1.2: Schematic showing brain regions involved in maternal behaviour. Brain regions (filled pink circles) where c-fos expression is significantly higher in lactating rats and mice compared to virgin rats and mice. Abbreviations: BnST = bed nucleus of stria terminalis, LS = lateral septum, MPOA = medial preoptic area, NAcc = nucleus accumbens (this is linked to the ventral striatum which is also indicated as important in the maternal brain circuitry), OB = olfactory bulbs, PAG = periaqueductal grey area, SON = supraoptic nucleus and VMH = ventromedial hypothalamus.

the MPOA does have links with parental behaviour in males) [72]. This could be due to the role of the MPOA in the regulation of prolactin release which is described above as essential in the onset of maternal behaviour [74, 76-78].

The MPOA is considered to play a central role in regulating maternal behaviour because both electrical and excitotoxic MPOA lesions disrupt most if not all components of maternal behaviour in rats [67, 79]. MPOA electrical lesions disrupt

maternal behaviour if performed pre- or post-partum in pregnant rats or in pup-sensitized virgin rats or before maternal behaviour onset in hormonally primed pregnancy-terminated rats [27, 79-82]. Excitotoxic MPOA lesions in rats disrupt maternal behaviour indicating that MPOA neuronal cell bodies are important for maternal behaviour, and are not just part of the pathways that pass through the MPOA to different brain regions [27, 83]. In addition, electrical stimulation of the MPOA enhances the display of maternal behaviour [79].

The bed nucleus of stria terminalis (BnST) is included in this section because it has been reported that MPOA excitotoxic and knife cut lesions which included the BnST, specifically the ventral part (vBnST), caused the greatest disruption of maternal behaviour [27, 67]. Specific vBnST lesions are also observed to impair expression of maternal behaviour, but only certain components, however this does highlight that the two brain regions may work together to regulate maternal behaviour [67]. Deficits in maternal behaviour caused by MPOA and vBnST lesions are unlikely to be caused by neuroendocrine changes because disruption of maternal behaviour still occurs in hormonally primed and pup-sensitized MPOA lesioned virgin female rats [27]. These lesions also do not result in other behavioural dysfunction which might indirectly affect maternal behaviour as normal sexual behaviour, locomotion, body temperature and body weight regulation are observed in MPOA/vBnST lesioned rats [27, 82, 83]. The BnST is part of the limbic system which is essential for controlling emotional output [84-87]. Thus the importance of the BnST may be in controlling the emotional state of the mother during the peri-partum period when there are dramatic hormonal and emotional changes [84].

Fos activation in the MPOA is higher following the maternal behaviour display in both mice and rats [9, 69, 88, 89]. Examination of expression of other IEGs in rats, namely Fos B and Egr-1, also show higher levels in the MPOA following display of maternal behaviour [69]. These IEGs have different patterns of expression during maternal behaviour exhibition; Fos was increased 2h following pup exposure and remained high during dam-pup interactions whereas Fos B expression significantly increased gradually as the duration of dam-pup interaction increased [69]. Egr-1 expression, by contrast, rose dramatically 2h after pup exposure but then decreased to basal levels following 4h of pup exposure [69]. The differences in Fos and Fos B expression patterns could be considered to be revealing short and long term maternal behaviour stimulation [69]. However, over time dam-pup interactions may change and the change in Fos-B expression may actually reflect these instead [69].

Higher Fos expression in the MPOA of mice was unaffected by ovariectomy, therefore the ovarian hormones are not the only important neuroactive substances controlling the neural circuits regulating maternal behaviour [88]. Furthermore, removal of the noradrenaline source in the olfactory bulbs (OBs) did not affect Fos expression in the MPOA in mice, so we may conclude that information other than olfactory cues can activate this neural circuitry [88]. Interestingly, it was hypothesised that MPOA activity is controlled by the 'maternal' state because in lactating rats neither anomsia nor ventral desensitization by anaesthesia (removal sensory input from nipples and ventrum hence preventing somatosensory cues from pups) has any effect on Fos MPOA expression compared to intact control rats [89]. These results suggest the MPOA could be a brain region that is part of an 'effector

mechanism' which controls maternal behaviour but does not require sensory or olfactory input to regulate it [89].

However this seems unlikely as Fos expression is higher in the MPOA of pup-sensitized maternal virgin rats compared to non-maternal virgins. Therefore rats that experience only olfactory and sensory cues to 'switch on' maternal behaviour show increased activation of the MPOA [70]. This elevated Fos expression in pup-sensitized maternal rats is greater than in rats exposed only to an inanimate object (marbles) emphasizing the necessity for all pup cues for MPOA IEG activation when hormonal influences are not available [70]. MPOA lesioned rats were unlikely to obtain pups where pups were a reward for using an operant bar press, but no difference was observed for food pellets in the same paradigm [79]. This suggests the MPOA is required to integrate the rewarding and reinforcing cues from pups to increase maternal behaviour in the primiparous rat [79].

The MPOA and vBnST areas project to many specific brain areas which are also activated during maternal behaviour display in the rat [90]. The main MPOA projections activated during maternal behaviour were to the medial hypothalamus (MH) and the lateral septum (LS) and are proposed to be involved in an inhibitory circuit which acts on these areas to reduce fear and anxiety towards pups [90]. vBnST projections that are activated during maternal behaviour include the ventral tegmental area (VTA), the retrorubral field (RRF) and the periaqueductal grey area (PAG) [90]. The VTA and RRF are linked to the with dopaminergic system involved in motivation, which indicates that the vBnST projections are important for the motivation to express maternal behaviour [90]. The projections to the PAG from both the MPOA and vBnST may also play an important role in pup retrieval behaviour as

the PAG itself projects directly to the trigeminal sensory complex, as does the RRF [90-92]. Lesioning of the trigeminal nerve in the lactating rat significantly disrupts most if not all oral motor maternal behaviour functions such as pup retrieval, licking and grooming [93]. This is probably due to the loss of sufficient somatosensory inputs to the snout from the pup to cause the jaw opening relex to pick up the pup [93].

Examination of ^{14}C-2-deoxyglucose (2-DG) uptake is an indication of activity of nerve terminals and hence provides information about inputs into specific brain regions [28]. 2-DG is a radioactively labelled glucose molecule with hydrogen replacing one of its hydroxyl groups; this means it cannot be broken down any further by enzymes hence when taken up into the active presynaptic neuron for energy it remains there and levels can be read by autoradiography. If investigated alongside Fos expression (that reflects post synaptic activity) this uptake enables researchers to interpret the nature of the input-output relationship of that specific brain region during expression of a particular behaviour [28]. In post-partum, pup-sensitized or hysterectomised maternal rats, it was observed that increased 2DG levels correlated with increased post synaptic activity in the MPOA compared to non-maternal controls suggesting an excitatory input-output relationship and highlighting the importance of the MPOA in maternal behaviour control irrespective of the trigger for maternal behaviour onset [28].

1.1.3.2 Amygdala

Like the BnST, the amygdala is part of the limbic system and is crucially involved in the control of emotion [94-97]. As previously described the peri-partum period is an intense time of hormonal and physical changes along with new environmental demands, i.e. the caring for offspring, thus it is essential for the emotional state of the

mother to be stable. This does not only benefit the mother in her ability to care for the offspring but can also have important consequences for the mental and physiological development of the offspring as well. Therefore the function of the amygdala in control of emotion, especially fear and anxiety, could explain why it is linked with an important role in the regulation of maternal behaviour regulation.

Virgin rats are fearful of pups thus it is hypothesised that the amygdala during pregnancy and lactation acts to decrease fear and anxiety to allow maternal behaviour [29]. This was deduced because whole amygdala lesions reduced withdrawal from pups and fear display in the open field test [79]. Fos activation is also observed to be significantly higher in the amygdala following pup exposure in pup-sensitized maternal mice [88]. However, the amygdala is composed of many different sub-regions with various functions. Therefore although whole amygdala lesions were observed to facilitate maternal behaviour it was proposed this was due to the removal of the inhibitory stimulus from medial amygdala (MeA) to the MPOA via the BnST [79]. Further evidence that the MeA specifically inhibited maternal behaviour was that Fos expression was increased in the MeA of pup-exposed non-maternal virgin rats compared to pup-sensitized maternal virgins [70].

The MeA receives a direct input from the OBs and relays the information to the MPOA and BnST; anosmia in virgin rats is known to reduce pup-sensitization latency, so the MeA is considered to receive these aversive olfactory stimuli from pups and inhibits maternal behaviour [30, 70]. MeA lesions not only significantly reduce Fos expression in brain areas stimulated in pup-exposed but non-maternal virgin rats but, along with lesions of the anterior hypothalamic nucleus and ventromedial hypothalamic nucleus, lead to stimulation of maternal behaviour in non-

maternal virgin rats [98]. Other studies have also observed this disinhibition of maternal behaviour, including pup retrieval and general pup interaction, in virgin rats following MeA lesions [89, 99, 100]. Thus, the MeA works to inhibit maternal behaviour in virgin female rats and this is proposed to be overcome by the hormonal changes during pregnancy to allow normal maternal behaviour expression at parturition in the lactating rat [9].

Nonetheless, Fos expression was also specifically elevated in the MeA in the lactating rat re-exposed to pups after 3 days of separation [89]. Anosmia in lactating rats resulted in lower Fos expression in the MeA indicating that the MeA may be important in the interpretation of olfactory stimuli from pups, and enabling maternal behaviour during lactation [89]. 2-DG was positively correlated with Fos in the MeA in post-partum rats but negatively in pup-sensitized virgin rats. In pup-sensitized virgins, increased Fos activation with decreased 2-DG activity in the MeA suggests that the pup-sensitization process decreases the inhibitory input to the MeA thereby disinhibiting projections to the MPOA and creating an excitatory input-output relationship with the MPOA [28]. In post-partum rats it is speculated that sensory information from uterine contractions during parturition helps overcome this inhibitory input to result in an excitatory one [28]. This is because Fos and 2-DG activity was significantly increased in post-partum rats in the MPOA, MeA, BnST and ventromedial hypothalamus (VMH) compared to controls (non-maternal virgin rats) and these areas show higher Fos expression in conjunction with birth canal stimulation [28]. Also, hormones are unlikely to be the cause of the change in the input nature because pregnancy-terminated rats, which experience the hormonal changes of pregnancy until surgery but not parturition, do not display a change in 2-

DG activity compared to controls [28]. Therefore, during the virgin state the MeA works to inhibit maternal behaviour but once suppressed allows maternal behaviour expression at parturition, the MeA appears to help maintain normal maternal behaviour during lactation.

Lesions of the cortical part of the amygdala also disinhibit maternal behaviour in virgin rats [89, 99]. Furthermore, olfactory desensitization in the lactating rat resulted in lower Fos expression in the cortical amygdala after pup exposure. This indicates that that, just like the MeA, the cortical amygdala works to interpret olfactory information to help regulate maternal behaviour [89].

The olfactory processing by the MeA and cortical amygdala are not the only parts of the amygdala that are linked with the regulation of maternal behaviour. The basolateral amygdala (BlA) is important for learning, memory and memory reinforcement, especially in relation to emotional and olfactory memories [89, 94, 101-103]. Thus it is suggested that the BlA is important for attainment of maternal memories and experience [89]. Fos expression within the BlA is observed to be significantly higher in lactating rats following pup interactions compared to lactating rats that had social contact with a conspecific or no pup/social interaction [89, 104]. Sensory desensitisations (anosmia and ventral desensitization by anaesthesia) lead to significantly lower Fos expression in the BlA of lactating rats during pup interactions [89]. Furthermore the level of Fos expression was dependent in the BlA on the type of pup interaction the lactating rat experienced; exposure to pups in a box increased Fos in dams compared to those who just interacted with the box but highest expression was observed in dams that had physical contact with pups [89]. Fos expression within the BlA was higher in experienced lactating rats (i.e. that had prior exposure to pups)

than inexperienced lactating rats upon re-exposure to pups whether the pups were in the cage or a box [104]. No changes in Fos expression were observed in the MeA, cortical or central amygdala (CeA) [104]. Therefore the BlA activation during the initial pup interaction is proposed to be essential for the acquisition of maternal memory or experience necessary for the following pup exposures to initiate maternal motivation to care for the pups.

One final region of the amygdala important for maternal behaviour regulation is the CeA. The CeA, like the BlA, is important in emotional and olfactory memory processing as well as memory reinforcement [94, 104]. Sensory desensitization was also observed to result in significantly lower Fos activation during pup interactions in lactating rats compared to normal lactating rats [104]. This indicates that like the BlA, the CeA is important in processing sensory information about pups to create maternal memories essential for maternal motivation. However, the CeA is also known to be important for the control of emotional behaviour especially in short term cue specific responses (whereas the BnST is more involved in the long term non cued consequences) [84, 105]. Thus, the CeA may also be involved in controlling emotional responses, along with the BnST, to enable normal maternal behaviour expression.

The MeA and cortical amygdala therefore work to inhibit maternal behaviour in the virgin rats however in the lactating rat the MeA, cortical, BlA and CeA work to integrate and process sensory and olfactory information for maternal experience or memory acquisition and control of emotions to enable maternal behaviour.

1.1.3.3 Paraventricular nucleus

Situated adjacent to the third ventricle in the anterior hypothalamus is the paraventricular nucleus (PVN, see appendix 3). The PVN is an essential region of the brain because it is able to produce and control the release of many neuroactive substances from its neurones simultaneously [106]. The PVN is made up of thousands of cells which can be divided into three main groups, magnocellular, parvocellular and 'autonomic cells', based on their morphology and projections. The large magnocellular cells comprise the hypothalamic-neurohypophysial system (HNS) which project to the posterior pituitary where they release OXT and vasopressin (AVP) to control and influence multiple functions including blood osmolality and physiological stress responses [106-108]. The 'autonomic cells' of the PVN carry mainly oxytocinergic and some AVP, projections to the brain stem and spinal cord which impact upon cardiovascular functions, analgesia, food and water intake, and gastrointestinal functions [106, 109-117]. Finally, the parvocellular neurons of the PVN produce a number of neuromodulators including corticotropin releasing factor (CRF), thyrotropin releasing hormone, vasoactive intestinal polypeptide, cholecystokinin, substance P, neurotensin, enkephalin, growth hormone releasing factor or angiotensin II [106, 110, 118-122]. Thus the parvocellular neurones of the PVN can influence body metabolism, body temperature and food intake [106, 109, 112-115, 123].

The parvocellular neurones also project to the median eminence where CRF acts on the pituitary corticotrophs to elicit ACTH response, along with AVP from the PVN, to initiate the secretion of glucocorticoids from the adrenal cortex into the bloodstream [107, 108, 124]. This system is known as the HPA axis and controls the

homeostasis of the body response to stress which the PVN, therefore, has a primary role in the controlling body's stress response [107, 125]. The HPA axis was initially thought to be dependent entirely on the parvocellular PVN neuron activity but the discovery that the magnocellular PVN neurones of the HNS can release OXT and AVP from dendrites lead to the indication the HNS can also impact upon HPA axis functioning [126-129]. Dendritic OXT or AVP release from magnocellular PVN neurones is proposed to influence the HPA axis by affecting both parvocellular and/or magnocellular neuron activity either directly or indirectly within the PVN [107]. Local release may also be able to diffuse out into the brain and affect other limbic brain regions in their response to stressors [107]. The PVN is known to have essential actions on nursing behaviour alone, but for the purpose of this thesis the focus will be on relating PVN activation to maternal behaviour in general (for reviews on the PVN and its role in lactation and parturition specifically please see [130, 131]).

As the PVN receives input from the MPOA, it was hypothesised to form part of the maternal behaviour circuitry [80, 132]. However, no effect on maternal behaviour was observed following knife cuts of the lateral PVN connections (i.e. destroying the MPOA to PVN pathway) and PVN radiofrequency lesions in lactation day 4 rats [133]. These PVN lesions significantly disrupt the milk ejection reflex indicating the PVN is important for nursing behaviour, but they are not necessarily involved in the pathway that the MPOA acts upon to enable maternal behaviour [80, 133]. Yet these lesions were made during the lactation period, so the PVN may play a role in the initiation of maternal behaviour [80, 133]. Indeed, electrolytic PVN lesions performed on pregnancy day 15 prevents the initiation of maternal behaviour whereas there is no effect if this is performed on lactation day 4 [134]. Kainic acid lesions on

lactation day 2 disrupted retrieving as well as nursing behaviour and these effects were probably due to kainic lesions destroying local cell bodies but not affecting fibre projections [132]. As only retrieval and nursing behaviours were disrupted, the PVN may only play a minor role in the regulation of maternal behaviour [132].

However the PVN does have a crucial role in the control of the HPA axis activity which during pregnancy and lactation is observed to be significantly altered [37]. Basal circadian rhythm HPA activity is significantly changed from early pregnancy with lower corticosterone and ACTH release in rats [37, 135]. By late pregnancy, HPA responses to both physical and emotional stressors are depressed but late pregnant rats have an increase in corticosterone release [37, 38, 137-139]. However there is also an increase corticosterone binding globulin levels which reduces amount of free and therefore active corticosterone in blood compared to non pregnant rats [37, 38, 135-137]. The depression of the HPA axis response during pregnancy is proposed to be essential for the fetus in protecting it from harmful excess glucocorticoid exposure [36, 138].

Altered HPA activity remains until late lactation however now basal activity is increased with greater secretion of ACTH and corticosterone observed but hyporesponsitivity to stressors remains [37, 135, 139]. The increased basal activity is induced by pups suckling and hence may be related to the necessicity of glucocorticoid actions on cells that produce milk [37]. The hyporesponsive HPA axis activity is proposed to be due to change in central processing of a perceived stressor rather than stimulation of ACTH and corticosterone release because ACTH and corticosterone secretion is still rapidly induced by suckling even after a period of separation during lactation period [37, 139]. During the post natal period, this reduced

HPA activity is proposed to be result in a reduced fear and anxiety phenotype required to enable maternal aggression (see section *1.2.2.3.1* for further discussion).

1.1.3.4 Periaqueductal grey

The PAG, as mentioned in the section on the MPOA and BnST, is directly linked to the trigeminal sensory complex which controls motor output of the body [90-92]. This important function of the PAG may be essential in regulating the motor output of maternal behaviour. The PAG receives direct projections from the MPOA and vBnST which show increased Fos activation during maternal behaviour display [90]. Lesions of the caudal PAG in the rat have shown to be important for kyphosis (upright crouching nursing behaviour necessary for optimal weight gain in pups), which is induced by suckling [140]. Fos activation in the caudal PAG is increased in rat dams by the suckling stimulus compared to dams anaesthetised in the perioral area, whereas Fos expression increases in the rostral PAG after display of retrieval and licking behaviour [140]. Lesions of the rostral PAG did not affect maternal behaviour or the ability to pick up and move pups but did impair the ability of the rat dam to release pups [140]. Therefore different PAG regions are important for the motor control of the specific maternal behaviour components (e.g. nursing posture) via their ability to process sensory information in the rat [140].

The PAG is also important in controlling specific motor responses to different forms of stressors, including offensive and defensive reactions [141-145]. Thus the PAG may be important for maternal aggression expression in response to an intruder which is threatening towards offspring survival (see section *1.2.3.3.3* for further discussion).

1.1.3.5 Nucleus accumbens

The nucleus accumbens (NAccs) is, like the BlA and CeA, is involved in the formation of emotional memories and associative learning [89, 146, 147]. Fos expression is significantly increased in the NAccs following pup exposure in lactating rats regardless of previous maternal experience; hence the NAccs function in the maternal behaviour circuitry was proposed to be involved in "maternal memory" [89, 148, 149]. Electrolytic lesions of the NAccs shell, but not the core, disrupt "maternal memory", which is defined as the process of retaining maternal experience [150]. Further to this Li and Fleming [150] reported that lesions of the shell of NAccs extended the time to retrieve all pups. This effect was consistent across a range of conditions from length of previous maternal experience, influence of hormones, and pre- or post-partum lesion, thus emphasising the importance of the NAccs in pup retrieval behaviour [150]. Therefore, owing to the NAccs functions in attention and motivational behaviour as well as memory formation, the lesions during the peri-partum period that disrupt retrieval behaviour could be a result of the lesion affecting the NAccs shell to retrieve maternal memories working to motivate the mother to perform pup retrieval behaviour [89, 104, 150].

1.1.3.6 Lateral septum

The situation of the lateral septum (LS, see appendix 2) means it is a focal point in the brain receiving inputs from the prefrontal cortex, entorhinal cortex, hippocampus, amygdala, hypothalamus and BnST and projecting to diencephalic and mesencephalic regions [151]. Thus the LS is involved in many brain functions including social behaviours, HPA activity, cardiac function, thermoregulation, spatial cognition and pain [151-166]. The role of the LS in influencing social behaviours means that it has a

crucial role in maternal behaviour. It is proposed that the LS regulates behavioural functions rather than directly mediating them i.e. it collates the sensory information the brain is receiving from various regions, working out its relevance and then relays the most relevant information to other regions which then mediate the correct behavioural response [151]. This means that the appropriate behaviour response is expressed in relation to the surrounding environment [151].

Lesions of the septum in mice produced profound deficits in maternal behaviour whereas lesions of the cingulate cortex or thalamus had little or no effect [167]. This was observed not to be a consequence of motor or motivational impairment in performing maternal behaviour but to result from the inability to inhibit all maternal behaviour responses other than the required one to deal with the pup situation [167]. Similar results were also observed in rats by Flannelly *et al* [168]. However, these lesions affected the whole septum which is made up of many distinct sub-regions; each of these may be involved in a more specific aspect of maternal behaviour. The LS, for example, is suggested to be critical only for the expression of one component of maternal behaviour, specifically maternal aggression, and this will be described in more detail in section *1.2.3.2* [67].

There are many brain areas involved in maternal behaviour, some with a general role, for example MPOA and BnST; others with a specific function in controlling exhibition of a specific component of maternal behaviour (e.g. NA and LS, see Fig 1.3). However, although these lesion and IEG studies highlight the importance of several brain regions in maternal behaviour, they do not provide information about what neuromodulators may be activating or inhibiting these brain areas to cause the expression of maternal behaviour. Whilst other maternal behaviours

can be induced in non-hormonally primed virgin female rats to levels similar to that of lactating or hormonally primed rats, maternal aggression is never expressed at equivalent levels without hormonal manipulation and therefore provides an excellent behaviour to examine if neuromodulators applied to rats are inducing the maternal 'state' [3, 4, 41]. Hence, this thesis decided to focus on maternal aggression and to investigate the effects of specific neuromodulators on its expression.

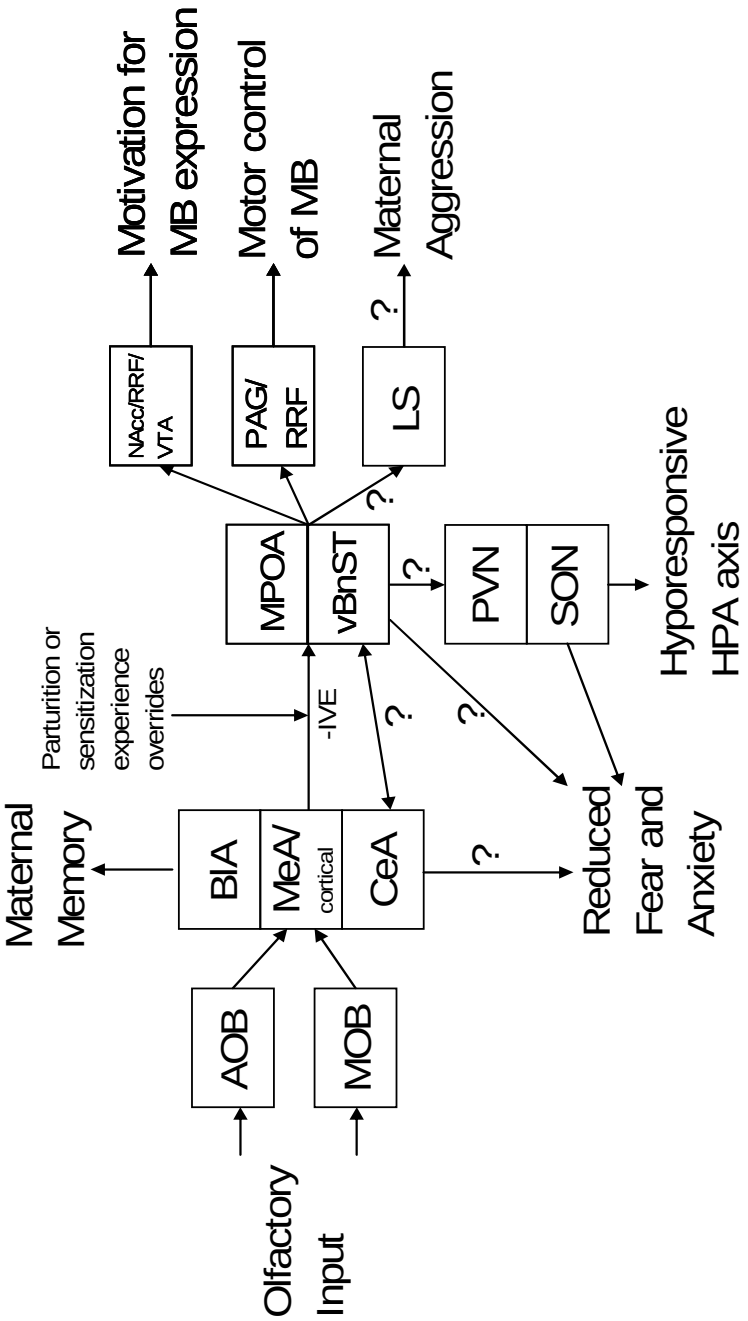


Figure 1.3: Schematic of maternal brain circuitry. Based upon Fig. 8 from Numan (2007)[9]. Flow diagram depicting how the brain regions linked with maternal behaviour may work together to control and regulate the different aspects of maternal behaviour expression. Although the functions of some regions in the maternal behaviour circuitry are established such as the BlA involvement in maternal memory and the NAccs controlling maternal motivation, it still remains unclear how maternal aggression is controlled within the brain (indicated by ? on the flow diagram). Also there are still questions about how the brain regions linked with controlling fear and anxiety under normal circumstances work during lactation to result in a reduced fear and anxiety phenotype. Abbreviations: AOB = accessory olfactory bulb, BlA = basolateral amygdala, CeA = central amygdala, MB= maternal behaviour, MOB= main olfactory bulb, MeA= medial amygdala, MPOA= medial pre-optic area, NAcc= nucleus accumbens, PAG= periaqueductal grey area, PVN=paraventricular nucleus, RRF= retrorubral field, SON=supraoptic nucleus, vBnST= ventral part of bed nucleus of stria terminalis, VTA= ventral tegmental area.

1.2 Maternal aggression

Maternal aggression was first reported as early as the 1940s when researchers described informal observations of wild and laboratory lactating animals of various species fiercely defending their offspring from intruders (cited in [169]). However it was not until the early 1970s that any systematic research was performed into maternal aggression in rodents with unequivocal results, and since then maternal aggression has been clearly defined in mice, rats, hamsters and pigs [6, 169-178]. Gandelman [169] reported that lactating mice displayed intense aggressive behaviour towards novel conspecific intruders of both sexes more than virgin female mice; this aggressive behaviour was expressed most strongly during early lactation (days 3-13) and had virtually disappeared by lactation day 21. In rats, the same pattern of aggressive behaviour display during lactation was observed; there was a peak just following parturition with a second peak around lactation day 9 before disappearing by lactation day 21 [168, 179]. Furthermore, in rats maternal aggression could be observed in pre-partum rats on pregnancy day 22 just hours prior to birth; the same time as maternal responsiveness is observed [180]. Female rats provide the best model (the one used in this thesis) to understand the control of maternal aggression for a number of reasons:

- Female rats are generally sociable and therefore are normally grouped housed in the laboratory with no aggressive behaviour displayed [181].
- Maternal aggression is generally only expressed by the rat during the peri-partum period whereas in mice and hamsters aggressive behaviour is observed outside of this period [3, 175].

- Maternal aggression in the virgin rat cannot be induced to lactating rat levels by pup-sensitization alone without hormonal manipulation, whereas mice only require a short pup exposure time before expressing maternal aggression [3, 182].

Research from mice will also be discussed to highlight the point that maternal aggression is important for offspring survival but is under diverse control in different species [27]. As maternal aggression is expressed during a short time span in the rat, normally from late pregnancy until mid-lactation, neural circuitry activation must only be temporary and consequently it is proposed that maternal aggression is induced by the dramatic hormonal changes that occur during the peri-partum period [12]. However, studies have shown that non-hormonal influences are also important for maternal aggression. The next sections will describe and discuss the influence of the different hormonal and non-hormonal factors on maternal aggression regulation and expression.

1.2.1 Hormonal influences on maternal aggression

Maternal aggression levels in pup-sensitized virgin female rats are always significantly lower than lactating rats; thus it was proposed that, as maternal aggression is a component of maternal behaviour, the changes in ovarian hormones (estrogen and progesterone) and the pituitary hormone, prolactin, during pregnancy are required to induce the full and vigorous display of maternal aggression [3, 173, 183]. Hence, the hormonal manipulations which induce maternal behaviour (discussed in section *1.1.2*) could also facilitate maternal aggression. Mayer and Rosenblatt [183] showed that maintaining high circulating estrogen levels during late pregnancy or elevating estrogen in pup-sensitized non-pregnant rats caused higher

maternal aggression than in rats where an estrogen rise was prevented, whether pregnant or not prior to pup exposure. This indicates that estrogen in pregnant rats works to heighten aggressive behaviour [56, 173, 183-185]. Furthermore once maternal behaviour was initiated (i.e. after rats were exposed to pups), all pregnant or pregnancy-terminated rats displayed maternal aggression regardless of whether they had done so before pup exposure and were more likely to show maternal aggression than pup-sensitized virgin rats [183]. The level of maternal aggression was lower prior to pup exposure than after it indicating that ovarian hormones may 'prime' the rat ready to express maternal aggression, but for a full display of aggression pup exposure is also required [183]. However, pregnancy-terminated rats (by hysterectomy and ovariectomy) treated with oil were not aggressive until 3-4 days following surgery when circulating estrogen levels would be low as a result of ovariectomy [183]. Thus other neuromodulators during the pre-partum period must have some function in maternal aggression onset; these could include pituitary hormones [183].

Hypophysectomy in rats on lactation day 4 has no effect on levels of maternal aggression observed on lactation day 9 or the normal maternal aggression level following pup removal or re-introduction [173]. Also, if hypophysectomy is performed earlier (i.e. on post-partum day 2), there is still no reported effect on maternal aggression across the entire lactation period [186]. These results suggest that circulating pituitary hormones are not necessary for post-partum stimulation of maternal aggression or to help maintain sensitivity to presence of the litter that modulates maternal aggression similar to the maintenance of maternal care, but do

not rule out a role in ‘priming’ the rat for maternal aggression expression pre-partum [173].

Maternal aggression can be induced in non-pregnant female rats by prolonged treatment with estrogen and progesterone, followed by progesterone withdrawal (as occurs in late pregnancy) whilst being exposed continuously to pups compared to pup-sensitized virgin female rats [56]. (Comparisons were only made between hormone treated pup-sensitized female rats and pup-sensitized only female rats, and only the attack behaviour was reported so it was not possible to conclude if the maternal aggression expression observed was similar to that in lactating rats [56]). This is not mediated by circulating pituitary hormones as the maternal aggression exhibited was unaltered in hypophysectomised female rats treated with the same hormonal course, and was significantly greater than in vehicle treated rats [56].

Progesterone withdrawal during late pregnancy, as for maternal behaviour onset, is proposed to be essential in enabling maternal aggression [56]. This was hypothesised because in two studies, the timing of the progesterone withdrawal in estrogen/progesterone treated in non-pregnant females resulted in different maternal aggression levels. Progesterone withdrawal 3 days prior to aggression testing resulted in a significant increase in maternal aggression prior to pup exposure but when progesterone was withdrawn only 1 day prior to testing no increase in maternal aggression was observed [56, 187]. During pregnancy, progesterone levels begins to decrease around day 20 (Fig. 1.1), so behavioural testing only one day after removal of progesterone may not be long enough for progesterone levels to have dropped fully to allow maternal aggression expression [3, 56].

Pregnancy termination on gestation day 19 by hysterectomy and ovariectomy did not prevent the ability of the rat to initiate maternal aggression even after delay of exposure to pups, however it was observed that maternal behaviour must have commenced before maternal aggression was displayed [184]. Thus it appears in the rat the ovarian hormonal experience of pregnancy induces long lasting changes which prepare the mother for maternal aggression [184]. However, non pregnant rats treated with estrogen and progesterone for 16 days to mimic pregnancy do not exhibit maternal aggression if pup exposure is delayed. Therefore estrogen and progesterone changes throughout pregnancy are not the only factors that prepare dams to be able to display maternal aggression and maternal behaviour [184].

In mice, the control of maternal aggression by ovarian hormones is not as crucial. Mice show little maternal aggression during pregnancy but there is a dramatic increase in intensity after parturition [188]. Estrogen levels rise immediately following parturition in mice before falling again and if mice are subjected to an ovariectomy during this time they exhibit more intensive aggression post-partum than intact mice therefore estrogen may prevent maternal aggression in mice [188, 189]. Furthermore, estrogen treatment in ovariectomised or lactating mice reduces maternal aggression thus indicating it is the estrogen surge just following parturition that inhibits maternal aggression in mice [188, 189]. Progesterone administration during lactation has no effect on maternal aggression in mice but during pregnancy progesterone enhances maternal aggression [188, 189]. Thus in mice estrogen works to inhibit maternal aggression during lactation whereas progesterone facilitates it during pregnancy, this is the opposite to effects observed in rats where estrogen is required to enhance maternal aggression during lactation and progesterone

withdrawal at late pregnancy is required for initiation of maternal aggression [56, 173, 183, 184, 186, 189]. In mice, sensory stimulation (as discussed in the next section) appears to be more important in inducing maternal aggression rather than the ovarian hormonal changes of pregnancy [188].

1.2.2 Non-hormonal influences on maternal aggression

Non-hormonal influences, including both sensory and olfactory cues, are important in maternal aggression expression in rodents, and the most important of these cues come from the pups. In mice, suckling experience for 24-48h is essential before maternal aggression develops [6]. Furthermore, pup-sensitized virgin female mice will display maternal aggression at levels comparable to lactating mice which is not the case for rats [3, 182]. Removal of pups in both rats and mice has been observed to diminish maternal aggression intensity [169, 179, 190]. Furthermore, the re-introduction of the pups to lactating mice or rats re-instates maternal aggression [6, 179]. Pup cues are essential in re-establishing maternal aggression and influence mice and rats very differently; for mice olfactory cues for pups are enough to re-instate maternal aggression whereas rats require both sensory and olfactory stimulation (for further discussion on the influences of pup on maternal aggression expression see the introduction for chapter 3).

Other non-hormonal factors that influence aggression include pup age, intruder age and sex and the test arena. Lactating rats who fostered older pups during early lactation displayed less maternal aggression than lactating rats with normal aged pups; furthermore if a dam towards the end of lactation was given young pups (aged 8 days), maternal aggression increased to early lactation levels [191]. However, these dams required at least 5 days of fostering before maternal aggression was tested.

Therefore it could be that pup independence, rather than age per se, influenced changes in maternal aggression intensity [191]. In mice, older juvenile intruders (20-40 days old) elicited a greater expression of maternal aggression towards them than young intruders (1-10 days old) [6, 188]. It was proposed that possibly hair growth, an age-related physical change, may be a causative factor in eliciting a higher level of maternal aggression [6]. Indeed, hairless mice were attacked less often than age-matched control mice so a change in physical appearance consistent with maturation in mice provokes a certain level of maternal aggression [6]. Maternal aggression was greater towards adult male intruders aged 35 or 45 days than those aged 55 or 110 days in lactating rats [192]. This could have been for a number of reasons, one is that the older males were much larger and maternal aggression is shown be greater against younger and smaller intruders [192, 193]. Another is that older males displayed aggressive behaviour towards the lactating rat and this may have decreased maternal aggression [192]. Pheromones can also change with age and it may be that older intruders produce pheromones that diminish maternal aggression; however, this is unlikely to be androgen based as testosterone levels are significantly decreased in 55 day old male rats compared to 110 day old rats, yet they elicit same similar levels of maternal aggression [192].

Sex of the intruder during early lactation in mice appears unimportant for the intensity of maternal aggression; however in later lactation male intruders elicit a greater level of maternal aggression displayed towards them than females [6, 169]. Primiparous mice and rats are as aggressive as multiparous animals, thus parity has no influence on the intensity of maternal aggression expressed [6, 7]. Lactating rats are thus far more aggressive in their home cage than in a novel setting hence a novel

environment can inhibit aggression but no difference was observed with different sizes of home cage [185, 192].

Pup-sensitized virgin female rats express low levels of maternal aggression if tested 9 days after becoming maternal, however if they are exposed to pups for at least 18 days maternal aggression is expressed at similar levels to lactating rats [172]. This prolonged exposure resulted in a pseudopregnant state, so hormone changes similar to pregnancy occurred, possibly enabling the display of maternal aggression [172]. Another point is that the initiation of maternal behaviour in pup-sensitized rats did eventually enable maternal aggression, while other studies have also observed an increase in maternal aggression in non-pregnant pup-sensitized rats compared to nulliparous controls, indicating that the experience of maternal behaviour can facilitate the onset of maternal aggression [174, 184].

The current view for the control of maternal aggression in the rat is that pregnancy 'primes' the brain ready for expression, but the onset of maternal behaviour by pup stimulation is required before the full suite of maternal aggression components can be initiated [12, 27, 184]. However, for mice hormonal influences appear to be unimportant and it is the suckling experience itself that controls maternal aggression onset [188].

1.2.3 Neural circuitry of maternal aggression

Many brain regions are involved in the maternal brain circuitry and they each have a complex role in regulating either general maternal behaviour expression or a specific maternal behaviour. The roles of specific brain regions in maternal aggression have been investigated recently, using the techniques described in section *1.1.3* for the

study of maternal behaviour, and the sections below will outline their possible involvement.

1.2.3.1 Medial preoptic area and bed nucleus of stria terminalis

Interestingly, although there is strong evidence through lesion and IEG studies that the MPOA and the BnST are essential for regulating and instigating the expression of most components of maternal behaviour, few studies have examined their effect on maternal aggression. Hence little is known about the potential roles of the MPOA and vBnST in maternal aggression [67]. Lesions in the MPOA and vBnST cause general disruption of maternal behaviour and it is proposed that while maternal aggression is primed by the hormonal changes of pregnancy in the rat, maternal aggression requires maternal behaviour onset before its initiation [174, 184]. Therefore, if the MPOA and vBnST are lesioned and this is observed to disrupt maternal aggression, it would be unclear if this was as a result of a direct effect on maternal aggression, or by reducing maternal behaviour the lesion indirectly disrupts maternal aggression. However, IEG studies demonstrate an elevation in Fos in the MPOA and BnST of the lactating rat and mouse during a maternal aggression test [Meddle *et al* unpublished, [12, 194]. The MPOA and BnST are therefore activated during maternal aggression display but it is not known if this reflects a role in stimulating maternal aggression. Future research examining the role of the MPOA and BnST in maternal aggression is therefore required.

1.2.3.2 Lateral septum

The LS is important in maternal aggression as Fos expression is observed to be significantly higher in the LS following maternal aggression display in lactating rats and mice compared to non-maternal virgin controls [12, 188, 194], Meddle *et al*

unpublished]. Lesions of the septal region significantly disrupt maternal aggression in lactating mice [168, 188]. However, these lesions also caused considerable impairment to maternal behaviour, hence the diminished maternal aggression could be due to no suckling stimulation from pups which is essential for normal maternal aggression in mice [168, 195]. Yet, it has been observed that maternal aggression is sustained in mice that have had suckling experience followed by the continued presence of a non-suckling litter [168]. The mice with septal lesions did have suckling experience prior to surgery and their litter present throughout the experiment indicating that suckling stimulation alone cannot account for the decrease in maternal aggression [168]. Lesions of the septal region or even the LS alone have yet to be examined in the lactating rat.

1.2.3.3 Amygdala, paraventricular nucleus and periaqueductal grey area

Unlike the brain regions above (MPOA, BnST and LS), there is substantial evidence from Fos and lesion studies that the amygdala, PVN and PAG are crucial to the regulation of maternal aggression. The reasons these three brain regions may be crucial to maternal aggression are related to their essential roles in the control emotions and the HPA axis.

1.2.3.3.1 Paraventricular nucleus

PVN expression of Fos and Egr-1 in aggression tested lactating mice was both greater compared to untested lactating or virgin mice [12, 194, 196]. In lactating rats that have undergone an maternal aggression test, Fos synthesis in the parvocellular and magnocellular PVN is also significantly greater compared to control lactating rats [188], Meddle *et al* unpublished]. Electrolytic lesions of the PVN in the lactating rat significantly impair maternal aggression [197]. The lesion causes no deficits in pup

care or in the duration of sniffing the intruder, thus there is no change in ability to perform motor actions or social interaction which emphasizes that the disruption is to maternal aggression alone [197]. However, PVN kainic acid lesions were observed to have no effect on maternal aggression in the lactating rat, indicating it is the projections through the PVN which are important in the control of maternal aggression [132]. Ibotenic acid administration into the PVN destroys only the parvocellular division which projects to limbic and autonomic brain regions [198]. Ibotenic acid lesions to the PVN of the lactating rat made during early lactation resulted in an increase in maternal aggression, and no change was observed if the lesion was performed on lactation day 18 when maternal aggression is normally low [198]. This indicates that the parvocellular division of the PVN projections are important for maternal aggression inhibitory control during the time period when maternal aggression is at its highest [198]. Therefore, the PVN appears to be important in the control of maternal aggression early on in lactation but has little or no role later on [188, 198].

Research has shown that the pre- and post-partum period is generally associated with a reduced fear and anxiety profile in the mother [29, 31-33, 138]. The PVN has direct influence over the HPA axis, the responses of which as described in section *1.1.3.3* are observed to be down regulated during the peri-partum period [107, 199-201]. During the postnatal period, the dampening of the HPA axis response was thought to reduce fear and anxiety allowing the dams to express maternal behaviour towards pups (a fear inducing stimuli to virgin rats) and also provide protection for pups to a normally fear inducing stimuli [29, 42, 202, 203]. Evidence of this theory was a reduced corticotropin releasing hormone (CRH) activity, which along with

AVP normally activates the HPA axis, in the hypothalamus during the peri-partum period [202]. Furthermore if CRH is administered ICV to lactating mice levels of maternal aggression are significantly decreased [204]. This is also observed if urocortin 1 and 3, CRH related peptides, were given ICV to mice [205].

However, there is substantial inconsistent evidence with this theory. Firstly, a reduced fear and anxiety phenotype is not always observed with normal maternal aggression expression indicating a reduced fear and anxiety phenotype may not be necessary for heightened aggression [15, 206-208]. This could be due to differences in laboratory conditions, experimental procedures or strains of species [202]. Secondly, the blunted response of the HPA axis is maximal during the second week of lactation when maternal aggression intensity is normally decreasing [137, 202, 209]. Also adrenalectomy or hypophysectomy, which increase CRH levels in the brain, do not affect maternal aggression [173, 202, 210-213]. Finally, maternal aggression and anxiety return to virgin rat levels following removal of pups but the blunted HPA axis response requires a far longer period of pup separation to return back to normal [188, 209, 210, 214, 215]. Thus, the actions of the HPA axis do not link clearly with maternal aggression and extensive research is required to fully understand how maternal aggression is controlled and whether it is connected to the reduced fear and/or blunted HPA axis phenotypes. One possible theory is that during the pre-partum period the blunted HPA axis ‘primes’ the mother to be able to respond to her litter which was previously fear inducing on the day of parturition but thereafter fear, anxiety and aggression expression in the dam are all controlled by input from the pups.

1.2.3.3.2 Amygdala

Specific regions of the amygdala have been identified to be involved in maternal aggression by IEG studies. Fos expression is greater in the MeA and cortical amygdala of lactating mice following the display of maternal aggression [12, 194]. Egr-1 expression was observed to be significantly elevated in the MeA, CeA and BlA of lactating mice tested for maternal aggression [196]. In lactating rats, Fos expression is observed to be greater in the MeA and CeA during maternal aggression display than in control lactating rats [188], Meddle *et al* unpublished]. To my knowledge, few if any lesion studies have examined effects of amygdala lesions on maternal aggression in lactating rats or mice [188]. Research instead has focused on neuromodulator control of maternal aggression in the amygdala which will be discussed further in the sections *1.4-1.7* below which outlines the possible roles of different neuromodulators in the regulation of maternal aggression.

Specific regions of the amygdala, as described in section *1.1.2.2*, have differing functions. The MeA processes olfactory information so could be essential in the detection of a threat to the pups and therefore enabling maternal aggression in response to an intruder. The amygdala may also indirectly enable maternal aggression due to its involvement in the regulation of fear and anxiety. Another way the amygdala may indirectly allow maternal aggression is through its direct projections to the PVN which are observed to directly influence the HPA axis response to particular stressors [124]. The CeA exerts an impact upon the reactions to systemic stressors for example body trauma or inflammation whereas the MeA reacts to noise and restraint stressors [216-219].

1.2.3.3.3 Periaqueductal grey

Fos, pCREB and Egr-1 all show significantly greater expression in the PAG following maternal aggression display in rats and mice [12, 188, 194, 196], Meddle *et al* unpublished]. Lesions of the caudal PAG were observed to result in a higher level of maternal aggression in lactating dams compared to sham controls [140].

The PAG is an area crucial to regulation of the stress response, the descending pathway of pain and in general sensory processing [141, 220]. In response to stressors, different sub-regions of the PAG are essential in controlling particular stress responses. The dorsolateral region of the PAG is important in active physical responses (i.e. flight and fight) to psychological but escapable stressful situations and thus works to control motor activity, circulatory activity and increased vigilance necessary for the response [141-145]. The lateral PAG controls the active physical response to physical stressors [141]. The ventrolateral PAG region controls the passive emotional coping response in situations where the stressor is inescapable, physical or psychological [141, 143-145, 221]. Passive coping strategies include decrease motor activity, slowing of the heart rate and decreased vigilance. The passive response controlled by the PAG is also proposed to be important for recovery a from the stress response [141, 221]. These defensive reactions controlled by the PAG are proposed to be influenced heavily by GABAergic neurotransmission but serotonin, opioids, neuropeptides, histaminergic and excitatory amino acids have also been observed to have an impact [222]. Thus the PAG, like the PVN, is essential to cope with emotional stressful situations especially the motor component. Thus, a harmful intruder when looking after young may be a stressor for the dam which

requires the action of the PAG to initiate motor aspects of maternal aggression to protect her young.

1.2.3.4 Olfactory bulbs

The OBs process olfactory information from the animal's environment. The OBs has a clear link with maternal behaviour, as described previously in section *1.1.1*, where the olfactory information received from pups is relayed through the OBs to the MeA to inhibit the expression of maternal behaviour (see Fig 1.4) [4, 30, 70].

However, the OBs must also play a crucial role in the control of maternal aggression. Fos expression is observed to be significantly greater in the OBs of aggressive lactating rats compared to non-aggressive lactating rats [Meddle *et al*, unpublished]. Furthermore, lesioning the OBs or removing the olfactory cues from pups or intruders disrupts maternal aggression expression in lactating mice and rats (see introduction of chapter 3 for more detail) [181, 188, 223-225]. In virgin rats, the olfactory the information works to inhibit maternal behaviour but once maternal behaviour is expressed in lactating rats the olfactory information from pups is now salient and works to enhance maternal aggression.

1.2.3.5 Supraoptic Nucleus

The supraoptic nucleus (SON), along with the PVN, is the brain's major source of OXT and AVP which, as described in sections *1.4* and *1.5* respectively, are two important neuromodulators with strong links to maternal behaviour and maternal aggression [226-228]. The SON, like the PVN, is already established to be important for the normal functioning of lactation physiology [229-236]. However recent studies observed that Fos expression within the SON is significantly greater in lactating rats tested for maternal aggression than in controls indicating that the SON does play a

role in maternal aggression [Meddle *et al* unpublished]. Whether this is due to its role in systemic OXT and AVP release or a direct influence over behaviour expression remains to be established and is the subject of current investigation.

Although more extensive research is required into how these brain regions link or regulate maternal aggression, it is clear that maternal aggression is closely linked with the PVN, PAG and amygdala; all of which are involved in fear and anxiety circuitry. It is proposed that the decline in fear associated with the postpartum period could enable the increased aggression [15, 27, 203, 237-240]. Research is now focusing on how these brain areas link together and influence each other through neuropeptides and neurotransmitters to allow expression of specific maternal behaviours, as discussed in section *1.2.1* it is clear that hormonal changes of estrogen, progesterone and prolactin alone do not regulate maternal aggression.

1.3 Neurochemical control of maternal aggression

Many different neuroactive substances are proposed to be involved in maternal behaviour and maternal aggression regulation from neurotransmitters to hormones to neurosteroids. The experiments in this thesis focus on OXT, AVP, allopregnanolone and GABA. All have different actions in the brain and the next sections of this chapter will examine how their roles in the brain may be linked with maternal behaviour, maternal aggression and each other.

1.4 Oxytocin

OXT is so named after the Greek for ‘quick birth’ because of its involvement in the parturition process [131, 227]. OXT is nonapeptide hormone whose structure

consisting of a nine amino acid sequence (Cys-**Tyr**-Ile-Gln-Asn-Cys-Pro-**Leu**-GlyNH_2) with a sulphur bridge between the two cysteine residues; this is highly conserved across phyla suggesting that evolutionarily it is an important neuropeptide in the brain [227, 241]. So far only one OXT receptor (OTR) has been sequenced and it is a member of the class 1 G-protein coupled receptor family [131, 227, 242]. OTRs are distributed widely throughout the brain; in the rodent OTRs are located mainly in the OBs, tubercle, neocortex, endoprirform, hippocampal formation, central and lateral amygdala, BnST, NAcc and VMH [227, 243, 244].

In the periphery, OXT has sexually dimorphic functions; in females it acts upon the uterus and mammary gland to control parturition and milk ejection respectively [106, 131]. In males, OXT works on the testis muscle to contribute to ejaculation [106, 131]. Peripheral OXT also affects the heart and cardiovascular system where it reduces heart rate and arterial blood pressure [131, 245, 246]. The kidney is another peripheral organ site where OXT acts as a non hypertensive natriuretic to regulate blood osmolarity [131, 247-250]. In addition, OXT acts directly on the heart to elicit the release of atrial natriuretic peptide [131, 251, 252]. Thus, OXT has many crucial functions in both males and females when secreted into the periphery. However once OXT is secreted into the blood it does not easily cross the blood-brain barrier therefore blood and cerebrospinal fluid OXT levels can be independently regulated, but often central and peripheral secretions reflect each other [127, 131].

In the brain, the main sources of central OXT release are the dendrites and somatas of magnocellular neurons of the PVN and SON nuclei uncovered by immunohistochemical studies [227]. Dendritic OXT release by the magnocellular

PVN neurones occurs in response to a variety of stimuli from dehydration to suckling to stressors to drugs [128, 131, 253]. In response to a specific stimulus, the PVN magnocellular neurones display a distinct firing pattern to cause a specific pattern of OXT secretion [131]. For example, during lactation OXT is secreted in a pusatile fashion following each large synchronous burst of firing of OXT neurones, whereas hyperosmolarity causes a small increase in firing rate to result in a small rise in OXT secretion [130, 131, 254, 255]. OXT is observed to help regulate analgesia, thermoregulation, feeding, motor activity, cardiovascular activity and social behaviours from sexual to maternal to cognition [109, 131, 152, 200, 227, 256-276]. The involvement of OXT in the regulation of so many social behaviours and its importance in lactation and parturition highlights why OXT could be essential for the control of maternal behaviour, a period of important social interaction between mother and child essential for both mother and the future wellbeing of the offspring [227, 265, 277]. In the next few sections below, the evidence from rodent and human research on the role of OXT in these behaviours, namely affiliation, social memory and recognition, anxiety, stress will be discussed and related to the peri-partum period.

1.4.1 Affiliation

Affiliation is the creation of a bond between two individuals [227, 228]. In the laboratory this is generally studied through pair bonding, sexual behaviour or parent-child interactions [227, 228]. Pair bonding has been particularly studied in voles because prairie voles form a monogamous relationship for life allowing comparisons to the promiscuous montane vole [227, 228, 265, 277, 278]. Central but not peripheral OXT infusions to an ovariectomised female prairie vole facilitates pair

bonding formation independent of mating (normally required) whereas pre-treatment with an OXT antagonist prevents pair bond development [227, 277-279]. Furthermore, the density of OTR is greater in the LS, BnST and NAccs (areas involved in the reward circuitry) of the monogamous vole compared to the promiscuous vole species [227, 265, 279]. In male prairie voles, central OXT infusion also facilitates pair bonding but at a much higher dose than AVP and this facilitation can be blocked by an OXT antagonist [278]. OXT administration to male prairie voles during their neonatal development period facilitated pair bonding in adulthood [280].

If pair bonding has a similar control mechanism as the parent-child bond, then one would expect aggressive expression to be higher in protection of partner [265]. Indeed, if female prairie voles cohabit with a male they display more aggression and less affiliation towards a novel female in a neutral test area [281]. Aggression remains high in females ovariectomised prior to cohabitation with males, indicating that sexual experience is not required for aggression and that the formation of a pair bond alone is enough [281]. Female prairie voles treated with OXT within 24h of their birth expressed more aggression and less social behaviour towards a novel female after cohabitation with a male in adulthood indicating neonatal OXT exposure can help the development of the mate-defence component of a pair bond [282].

In sheep, unlike rats and mice, ewes will form a specific olfactory memory of their young and are able to recognise them within 2h of birth [277]. Central OXT infusion in hormonally primed sheep facilitates this bond and OTR mRNA is increased in the granule cell layer of the OBs which regulates the formation of

olfactory memories [277]. In sheep, therefore, OXT in the brain helps to create a strong mother-infant bond by facilitating olfactory memory [277]. In humans, mothers are able to recognise the smell of their own infants within 30 min of their birth; so far though this has not been linked to OXT [277]. Yet, OXT levels do rise within the circulation of a mother whilst she is breastfeeding to cause milk ejection indicating there may be a connection [283]. Intranasal OXT administration to humans has been observed to increase trust and also decrease stress responses in a stressful situation [283]. These results suggest that like in rodents and sheep, OXT is important for creating bonds between individuals and therefore could be important for the development of the mother-child bond which is essential for offspring survival and adulthood social behaviour.

1.4.2 Social memory and recognition

Social memory and recognition enable individuals to distinguish between familiar and unfamiliar conspecifics allowing the display of appropriate behaviour towards them including the formation of social attachment [227, 283]. In rodents, the main stimulus for social memory and recognition are olfactory cues, mediated by the accessory and main OBs [227, 283]. In primates, auditory and visual cues also play important roles [227]. OXT is viewed as important for social recognition in both sexes because OXT KO mice, both male and female, display impaired social recognition, even after repeated trials [284, 285]. This is not due to olfactory or spatial memory impairment as OXT KO mice perform just as well as wild type mice in olfactory or Morris water-maze tasks [284]. OXT infusion into the lateral ventricles of the male OXT KO mice was able to rescue their social recognition impairment [284]. Moreover OXT antagonist administration to wild type male mice

impeded their social recognition abilities [284]. In female mice, administration of OTR antisense DNA to the MeA resulted in lower OTR expression and social recognition impairment [286]. ICV OXT antagonist infusion to female rats produced significantly impaired social recognition compared to vehicle treated females [227, 287]. OXT infusion into the OBs of male rats was able to facilitate social recognition; this effect involves noradrenaline as depletion of noradrenaline by 6-OHDA administration prior to OXT infusion prevented normal social recognition responses [288, 289]. Therefore, in male and female rodents OXT may facilitate social recognition by helping the information to be processed to create social memories, especially in the amygdala and OBs [227, 284, 290].

This olfactory learning may be taught to the pups by the mother's maternal behaviour during the neonatal period [283]. Paint-brushing, used to simulate maternal licking, whilst being exposed to a novel smell, induces pups to remember and have preference for that smell even in the absence of 'licking' [283]. Furthermore, the odour from the mother can be linked to non-social odours (peppermint) so that pups prefer the non-social odour when mother is not present [283]. ICV OTR antagonist prior to the learning phase of this paradigm prevents establishment of the association between the mother's odour and the non-social odour [283]. Thus, during development OXT plays an important role in learning social odours which could have important implications for how a rodent reacts in adulthood to social stimuli [283].

OXT administered subcutaneously or directly into the septal region to male rats decreased the social investigation time of a familiar juvenile rat during a second encounter indicating facilitation of social recognition [291, 292]. However, no effect

was observed with OXT antagonist treatment prior to the first encounter, but OXT injected into the lateral ventricles of male rats immediately after the first encounter facilitated social recognition and OXT antagonist treatment prevented this, leading to the proposal the OXT was important for social memory acquisition not retention [227, 292]. OXT in humans has been shown to improve social memory for faces, significantly angry or neutral, when administered intranasally in both males and females after the learning task [293]. The amygdala has also been implicated in control of facial recognition in humans with decreased amygdala activation following OXT administration to male humans when looking at angry or fearful faces [227]. OXT therefore in rodents and humans appears to be important in processing information in the amygdala and OBs to create memories which help with social recognition, even during development.

1.4.3 Stress and anxiety

All mammals face aversive stimuli which affect their normal homeostasis; in general there are two mechanisms which control the animals behavioural, autonomic and neuroendocrine responses [107]. These are the sympatho-adrenergic system which works to control the immediate active reactions to a threatening stimulus and the HPA axis which regulates the long term passive responses [107]. If there is uncontrollable, long lasting stimulation of the HPA axis then a maladaptive response may result in emotional disorders such as anxiety or panic [107]. One of the main features of the HPA axis is the PVN which is one of the main sources of OXT and AVP and research has observed a higher release level of these two neuropeptides whilst rodents perform stressful tasks [107]. Rats who experienced social defeat had a greater OXT release in the SON but no change in the PVN, however a forced swim

test caused a greater release in both the SON and PVN [107, 294-296]. Both of these tests are able to activate the HPA axis as demonstrated by increased ACTH and corticosterone levels in rats after testing compared to before [107]. Thus the release of OXT (and AVP) from the SON and PVN in response to stressful stimuli can modulate ACTH and glucocorticoid release hence they can impact HPA axis activation [107, 297]. OXT reduces ACTH and glucocorticoid release to negatively modulate the HPA axis to decrease the effect of stressful stimuli, whereas AVP works in the opposite way [35, 107, 298, 299].

In ovariectomised estrogen-treated female rats, ICV OXT infusion resulted in significantly lower plasma corticosterone levels following a noise stress compared to saline infused rats [300]. OXT treated female rats spent significantly more time and made more entries to the open arms of the EPM, thus displaying reduced anxiety behaviour; the same effects were observed in ovariectomised estrogen-treated mice [299, 300]. Ovariectomised estrogen-treated female rats infused with OXT prior to a restraint stress test showed significantly lower release of ACTH and corticosterone and lower Fos expression in the PVN, LS and dorsal hippocampus subregions [298, 300]. The fact estrogen is required in these paradigms is interesting as towards the end of pregnancy estrogen levels rise dramatically (Fig. 1.1), so high circulating estrogen levels may potentiate the important anxiolytic effects of OXT around parturition especially within the LS where higher OXT binding was correlated with estrogen induced anxiolysis [299].

These anxiolytic effects of OXT are not restricted to females as direct OXT administration to the PVN of male rats resulted in more time on and more entries into open arms of the EPM thus a lower anxiety profile than vehicle injected rats [301].

OXT release was higher in the CeA of male rats during a forced swim test [302]. Furthermore, infusion of OXT into CeA of these male rats reduced floating time and increased swimming time suggesting that the activation of OTRs in the CeA works to increase a passive stress response [302]. In male mice, ICV OXT administration reduced anxiety expression in the four-plate test, EPM and stress-induced hypothermia [303].

It is well documented that the peri-partum period results in a lower anxiety profile in mothers and during this time the OXT system changes dramatically hence OXT may regulate the anxiolysis and lowered stress response of motherhood [29, 31-33, 35, 297, 304-307]. In response to a mild psychological stressor (airpuff), late pregnant and parturient rats have significantly attenuated stress-induced release of ACTH and corticosterone compared to virgin female rats [35]. However, OXT antagonist administration did not alter ACTH or corticosterone release in response to a stressor in late pregnant or parturient rats [35]. ICV infusion of OXT antagonist to virgin female rats increased basal and stress-induced corticosterone but had no effect on the levels in pregnant or lactating rats [40]. Thus during the post-partum period, the reduced HPA axis activity to stressors is not mediated by OXT, but OXT does modulate anxiety behaviour [40]. Administration of an OXT antagonist reduced anxiety behaviour on the EPM in pregnant and lactating rats [40]. In addition infusion of OTR antagonist into the caudal PAG of post-partum rats, where lesions are observed to be anxiogenic, significantly reduced the time spent in the open arms of the EPM compared to vehicle treated rats [308].

In humans, OXT has also been linked with emotional disorders including depression and personality disorders involving excessive aggression [309, 310].

More recently, the OXT system has been linked with autism where there are severe deficits in social behaviour including social coping and creating interpersonal relationships indicating the importance of OXT for normal social behaviour expression [283, 311-313].

OXT effects on emotional state are also linked with the post-partum period in humans [283, 314]. During breastfeeding plasma OXT levels are higher in mothers and these correlate with lower stress experience and negative mood than bottle feeding mothers who have lower plasma OXT levels [314, 315].

1.4.4 Oxytocin, maternal behaviour and maternal aggression

OXT is proposed to be important for maternal behaviour onset but not necessary for its maintenance; this is because ICV OXT administration can induce maternal behaviour to virgin female rats and an OXT antagonist given ICV can prevent maternal behaviour onset to pregnant rats, but ICV application of an OXT antagonist once maternal behaviour has been initiated has no effect [3, 44, 131, 316, 317]. Furthermore, OTR expression in the SON, BnST and MPOA significantly increases at parturition but within 12h post-partum has returned to virgin rats levels; these areas are as important in the regulation of maternal behaviour [307, 318].

In sheep, prior maternal experience caused higher levels of OTR mRNA expression in many brain regions including the PVN at parturition than in inexperienced ewes [319]. The higher increase in OTR mRNA in the PVN of experienced than in non-experienced ewes may contribute to the onset of maternal behaviour as inexperienced ewes take longer to demonstrate maternal behaviour and the PVN is the main source of OXT [319]. Thus OXT can contribute to increased maternal responsiveness by inducing greater OTR expression in the brain following

previous maternal experience [319]. OTR mRNA expression and OXT immunoreactivity are also observed to change dynamically over pregnancy, parturition and lactation in the rat [304, 307].

In terms of maternal aggression, there is evidence for an essential role of OXT in maternal aggression control, especially within the PVN; however currently it is unclear if OXT inhibits or facilitates maternal aggression. PVN ibotenic acid lesions and OXT mRNA antisense (reduces OXT synthesis) inhibited maternal aggression on postpartum day 5 [188, 198]. However, neither ICV injections of OXT just prior to testing or peripenduncular lesions (preventing suckling-induced OXT release) had any effect on maternal aggression [188, 267]. Yet, OXT secretion is increased within the PVN during maternal aggression testing in the lactating rat and OXT antagonist administration directly to the PVN inhibits maternal aggression [320]. Thus research does indicate a role of OXT in maternal aggression and further discussion of the importance of OXT in the regulation of maternal aggression can be found in the introduction for chapter 4.

OXT therefore is linked with maternal behaviour and maternal aggression although its role in their regulation is not as yet completely understood [3, 44, 304, 307, 314, 316-319]. The many functions of OXT in regulating social behaviour suggest OXT may be important during the peri-partum period especially for maternal aggression for numerous reasons. OXT is anxiolytic and the ‘maternal’ state is a time of reduced anxiety and fear thus OXT may work to enable maternal aggression by reducing fear and anxiety [35, 40, 314]. OXT also promotes affiliation, thus OXT may help to create the mother-infant bond enabling the full display of maternal behaviour [265, 277, 281-283]. Finally OXT helps with social recognition, therefore

in terms of maternal aggression it may enable lactating rat to distinguish intruder as foe and therefore express the appropriate behaviour towards them [227, 283, 284, 290, 293].

1.5 Vasopressin

AVP is also a nonapeptide and differs from OXT by two amino acids (Cys-Tyr-**Phe**-Gln-Asn-Cys-Pro-**Arg**-GlyNH_2) [131, 228]. Like OXT, the structure of AVP is highly conserved across phyla, in birds and reptiles vasotocin is its evolutionary equivalent [228]. The SON and PVN of the hypothalamic nucleus are the main synthesizers of AVP but AVP is also known to be synthesized in the MeA, BnST, and suprachiasmatic nucleus (SCN) neurones of the brain [228, 321]. The latter of these has projections which terminate in the septal area and release AVP leading to its involvement in many brain functions [321]. There are 3 types of AVP receptors (V1a, V1b and V2), all of which are seven transmembrane receptors [228]. Although all 3 are expressed in different specific areas in the periphery, within the brain generally only expression of V1a and V1b receptors are observed [228, 322]. V1a receptor autoradiography (examines the location of receptor by allowing binding to a radioactively labelled ligand) and ISH has uncovered V1a receptor distribution in various brain regions of the rat including the LS, BnST, VTA, PVN, OBs and amygdalostriatal (transition area between amygdala and striatum) which are known to be part of the maternal behaviour and maternal aggression circuitry [12, 28, 67, 70, 79, 80, 140, 148, 188, 194, 228, 323, 324]. Binding of V1b receptors in the rat brain has yet to be examined due to the lack of a specific radioactively labelled ligand [228]. However, ISH studies observe V1b receptor mRNA in many brain

regions including those implicated in maternal behaviour or maternal aggression circuitry namely the septum, PVN and OBs [228].

AVP has many different actions in both the brain and periphery to control multiple functions. Two main stimuli which cause systemic AVP release from hypothalamic brain regions are stress and changes in plasma osmolality. AVP is released from the PVN magnocellular neurones into the bloodstream via their terminals in the posterior pituitary in response to blood hyperosmolality [107, 108]. AVP acts as an antidiuretic agent in the kidney to initiate water retention and return plasma osmolality to normal [107, 108]. Stressful stimuli will activate the HPA axis causing the release of AVP synthesised in the parvocellular PVN neurons of the hypothalamus (along with CRH) resulting in ACTH release and thus glucocorticoids from the adrenal glands which controls physiological stress responses of the body [107, 108]. Like OXT, once AVP is released systemically, the blood/brain barrier prevents re-entry of AVP into the brain so systemic and central AVP levels, although they may be linked, can be independent of each other [108, 127].

Within the brain, AVP is released from the magnocellular neurones of PVN and SON through their neuronal somatas and dendrites meaning AVP is able to diffuse through the brain in the extracellular fluid and act on regions distal to its release point [108, 126-129]. The parvocellular cells of the PVN are known to release AVP centrally as their projections terminate in the PVV, BnST, MeA and SCN [228]. Together, these multiple means of AVP release indicate that local brain AVP levels can be carefully controlled and this could explain how AVP is involved in a wide range of brain functions, from cognition to social behaviour to circadian rhythm control in the brain [108, 325] (for full review see [228]). In the next

sections, AVP links with affiliation, social recognition, stress and aggression will be reviewed in the context of how these actions may be important to the peri-partum period.

1.5.1 Affiliation

Affiliation is clearly linked with AVP [227, 228]. Sexual partner preference is one bond research has observed is clearly linked with the AVP system; this has been especially studied in prairie voles due to their lifelong monogamous relationships they form [228, 326]. In male prairie voles, AVP plays a central role in affiliation because ICV AVP administration to males housed with an ovariectomised mate enabled formation of pair-bond even though no sexual experience had occurred; normally essential for pair bond formation [326]. When sexual experience has occurred, ICV infusion of V1a antagonist to male prairie voles was able to prevent formation of a pair-bond [326]. OXT and CSF did not have any effect in either paradigm [326]. ICV treatment with AVP (or OXT) in male and female prairie voles prior to nonsexual cohabitation induced a pair bond, since more time was spent with the vole from the cohabitation period than the novel vole during a partner preference test [278]. AVP given to male voles at a 1ng dose was more effective than OXT in creating this partner bond as OXT only became effective at a dose of 10ng [278]. In females, AVP (and OXT) only became effective at the 100ng dose [278]. In this experiment the partner preference could be prevented by pre-treatment with an OXT or an AVP antagonist in both males and females [278]. This indicates both AVP and OXT are important in pair bond formation but in males AVP is more influential.

The parent-child bond is important to study as abnormal parental care has lifelong consequences. Paternal care is not generally observed in the male rodent

hence research has focused on the prairie vole and California mouse, two species in which the male is actively involved in the raising of the offspring [228]. Paternal care has been attributed in these parental rodents to differences in the central AVP system in the brain [228]. In male prairie voles there is higher expression of AVP immunoreactive fibres and neurones in the LS and BnST than in female prairie voles. Furthermore castration results in lower paternal behaviour and AVP expression in these areas, indicating steroid hormones are involved in the regulation of paternal behaviour [228, 327]. Administration of AVP to sexually naïve male prairie voles can induce paternal care [228]. This was also observed to occur in the non-paternal or pup-aggressive meadow voles who are promiscuous and do not normally display paternal care [228, 328].

In California mice, AVP immunoreactivity is higher in the BnST, and V1a receptor expression is greater in the LS compared to that observed in the polygamous white-footed mouse (a non-paternal species) [329]. Thus for paternal care AVP is important for both male voles and mice [228]. For female rodents, the full role of AVP is still unclear in maternal behaviour. AVP release in limbic and septal brain regions changes dramatically over the peri-partum period but this release has yet to be examined in relation to maternal behaviour [228, 330].

1.5.2 Social recognition

The central AVP system is important for creating affiliation because of its links with social recognition [228, 278]. Brattleboro male rats are unable to naturally synthesise AVP and display impaired social recognition [331]. This impaired social recognition can be rescued by infusion of AVP into the mediolateral septal region of the Brattleboro rats [331]. Furthermore, microdialysis AVP V1a receptor antagonist

infusion into the mediolateral septal area of male Long Evans rats (that have normal social recognition skills) resulted in an impaired social recognition phenotype similar to the male Brattleboro rat under normal conditions [331]. In the Wistar rat (that also have normal social recognition abilities), application of an AVP receptor V1a anti-sense oligonucleotide into the septum reduced AVP binding and resulted in an impaired social recognition phenotype [332].

In the V1a receptor KO male mice, an impaired social recognition phenotype is observed but this is not due to an olfactory impairment as both wild type and V1a receptor KO mice could become habituated to a scented cotton wool ball [333]. A V1a receptor viral vector (to increase AVP binding) was able to rescue the social recognition impairment in the V1a receptor KO male mouse and enhance social recognition in the wild type if applied directly to the LS but not the MeA [334]. These studies indicates that the V1a receptor and LS are important in social recognition [334]. Others however have failed to see this social recognition deficit in V1a receptor KO mice but observe an olfactory one instead; they suggest that the V1a receptor is important in olfactory processing, and because the V1b receptor KO mice in their laboratory show social recognition impairment, it is the V1b receptor is involved in social memory retrieval for social recognition [228, 290].

AVP therefore is important in social recognition but again research has only focused on males so it is as yet unknown if AVP is as important in females and hence necessary to help with bonding during the peri-partum period.

1.5.3 Anxiety, stress and depression

AVP also plays a role in the control of anxiety behavioural responses and HPA axis functioning, which as described above in the OXT section is essential in controlling

the stress response [107, 200, 228, 335]. In male rodents, there is significant evidence for AVP in regulating anxiety. In male rats, AVP release within the PVN, LS and amygdala is significantly higher following water-maze and forced swim tasks respectively [336, 337]. AVP may differentially mediate active and passive components of the stress response, as application of an AVP V1 receptor antagonist directly into the amygdala and LS resulted in opposite effects on struggling (active) and floating (passive) without affecting overall swimming behaviour in the forced swim test [337]. AVP application to amygdala increased struggling but decreased floating and vice versa following AVP administration to the LS [337].

Both the V1a and V1b receptors are implicated in AVP anxiety regulation in rats and mice. In adult male rats, septal infusion of AVP V1a receptor antagonists resulted a lower anxiety profile on the EPM compared to vehicle or AVP treated male rats [321]. No difference in locomotor activity was observed between treatment groups therefore the change in anxiety expression was as a result of central AVP action [321]. I.p. V1b receptor antagonist administration in male rats also resulted in lower anxiety profiles on both the EPM and in the punished drinking paradigms [338]. In male mice, V1b antagonist administration also led to a lower anxiety profile in the light/dark and EPM (after experience of social defeat stress) compared to vehicle treated mice [338].

There is a difference of opinion about whether AVP affects anxiety specifically through V1a or V1b receptors as the antagonists used are not necessarily specific to one receptor so cross-reactions can occur [333]. KO mice therefore provide a more definitive model to test which receptor mediates AVP actions on anxiety [333]. Male V1a receptor KO mice display significantly less anxiety

behaviour in the EPM, open field or light/dark box compared to wild type male mice [333]. No difference was observed in length of time immobile during a forced swim test between KO and WT male mice indicating a lack of V1a receptors affects anxiety but not depression behaviours and the lower anxiety behaviour expression in the KO mice was not due to an effect on locomotor activity [333]. In WT male mice, V1a receptor overexpression in the LS by direct viral vector injection caused a higher anxiety profile on the EPM [334]. However, when V1a receptor KO male mice were injected the same V1a viral vector into the LS no change was observed in anxiety behaviour on the EPM, open field or light/dark box tests [334]. This could be due to the fact that these KO male mice have no experience of V1a receptor throughout their development or life and that gene replacement in adulthood in one specific brain region is not sufficient to rescue this loss [334]. No difference in anxiety behaviour has been observed in male V1b receptor KO mice compared to wild types, but V1b receptor antagonist treatment in male rats resulted in less time spent immobile (a neophobic behaviour) in the forced swim test [228, 334, 338]. Thus one could propose that the V1a receptor mediates anxiety whereas the V1b receptor regulates depression.

Female V1a receptor KO mice do not exhibit a lower anxiety behaviour profile on the EPM, open field or light/dark box test [339]. Thus, there may be a sex difference in anxiety control, with V1a receptors not being essential in female mice for anxiety expression. Although the brain regions in which AVP is released are the same in male and female mice, male mice exhibit more AVP positive cells and release more AVP in the BnST and MeA [339]. Furthermore male mice have denser AVP projections than females to the LS from the BnST and MeA [339]. In female

mice, therefore, as discussed above it may be the OXT system which controls anxiety [339]. Also implicated are progesterone and its metabolites as being important for emotional stability in females and this is reviewed in section *1.6* [339]. However, this does not rule out AVP in modulating anxiety in the female as dramatic changes are known to occur in AVP release around the peri-partum period and research has as yet to investigate fully the relationship between AVP and anxiety and stress in female rodents [228, 330, 339].

A link between AVP and anxiety and stress disorders in humans has also been observed. Plasma AVP levels are higher in depressed patients who have increased numbers of AVP positive cells in the PVN [340]. Also a single nucleotide polymorphism in the V1b receptor is observed to protect against major depression [340]. For anxiety disorders, elevated plasma AVP level has been observed in post traumatic stress disorder patients compared to trauma or non-traumatized controls and obsessive-compulsive disorder patients [340]. Plasma AVP levels were correlated with anxiety symptoms in healthy humans following an anxiogenic drug challenge [341]. Thus there is evidence for roles of the AVP system in emotional disorders in both rodents and humans. Although research in rodents has focused on the male it is beginning to examine the significance of AVP in females and its possible importance in emotional disorders of the peri-partum period.

1.5.4 Aggression

As with most research investigating AVP effects on social behaviour, the focus has been on male rodents in terms of aggression. Male aggression in rodents generally reflects territorial protection or social hierarchy (cited in [342]). AVP in male rodents has been linked with excessive aggression, especially when related to alcohol or drug

taking paradigms, but aggression development has been linked with early life experience [16, 23, 228, 342-345]. In mice, the type of paternal care can influenced the development of aggression in their offspring [343, 344]. Cross-fostering of white-footed male mice pups to male California mice (who display paternal care) results in higher aggression than non cross-fostered male white-footed mice [343]. Furthermore, male California mice pups cross-fostered to white-footed males display lower aggression levels and a lower percentage of positive AVP cells in the BnST [343]. If adult California male mice are manipulated to exhibit more retrieval behaviour, their male and female offspring are more aggressive and in male offspring a higher number of AVP expressing projections in the BnST are observed [344]. It is unknown if the change in female aggression is correlated with change in AVP expression as this was not investigated, but these results do highlight the ability of the type of parental care to influence and programme adulthood aggressive behaviour in mice [343, 344].

Maternal behaviour has also been linked with adulthood aggressive behaviour display in males and females [14-17]. Prolonged maternal separation affects adulthood anxiety the same way in males and females, however it causes a decreased in intermale aggression but an increase in maternal aggression [16]. Furthermore, these different aggressive behaviour displays could be linked with changes in the neuropeptides, OXT and AVP; in maternally separated female mice OXT immunoreactivity was lower in the PVN whilst AVP remained unchanged, whereas in maternally separated males AVP immunoreactivity was higher in the PVN but OXT was unaltered [16]. Thus, as before these two neuropeptides are sexually dimorphic in their actions on social behaviours [16, 339]. It is important to remember

that there may be strain or species as well as gender differences in response to negative and positive maternal behaviour influences [16].

AVP, like OXT, has many links with social behaviours important for the peri-partum period such as anxiety, social recognition and affiliation [228, 290, 330, 333, 334, 339]. However the effects of AVP on these behaviours have yet to be investigated comprehensively in female rodents around the time of birth. Thus, there is a new focus of research on trying to understand how AVP works with or in balance to OXT to help maintain and regulate maternal behaviour.

1.6 Progesterone and Allopregnanolone

1.6.1 Are the effects of progesterone on maternal behaviour mediated by progesterone alone?

Changes in progesterone levels in the brain through pregnancy and parturition have important implications for maternal behaviour and maternal aggression (Fig. 1.1) [3, 27, 44, 45, 50, 180, 346-349]. The specific effects of progesterone on the development of maternal behaviour and maternal aggression were discussed in sections *1.1.2* and *1.2.1* respectively. The question presently being asked by researchers is how is progesterone is acting in the brain to prevent the onset of maternal behaviour and maternal aggression?

Progesterone can act in two main ways to cause change in cell or neural functioning; the first is to activate intracellular progesterone receptors (hormone dependent transcription factors) by binding to them and hence influence genomic actions [350]. The second is to affect cellular functioning by progesterone itself or its metabolites binding to a membrane receptor [350, 351]. Dynamic changes in

progesterone receptor expression in the brain during pregnancy and lactation have been observed with higher progesterone receptor expression in the anteroventral periventricular nucleus of the preoptic area, MPOA and VMN regions on pregnancy day 21 than pregnancy day 3 in Charles River CD rats [350]. By post-partum day 3, progesterone receptor expression was significantly lower in the AVPV, MPOA, VMN and ARC than pregnancy day 15 or 21 [350]. In Sprague Dawley (SD) rats, higher progesterone receptor expression was observed in the MPOA of pregnant and parturient females compared with virgins in proestrous [352]. Progesterone mRNA receptor expression was significantly higher in the MPOA, hypothalamus and temporal cortex on pregnancy day 15 in SD rats compared with pregnancy day 21 [353]. These areas, MPOA and hypothalamus especially, are proposed to be involved in the maternal behaviour circuitry, therefore progesterone may act at one or more of these sites to inhibit maternal behaviour [350]. The low levels of progesterone receptor in these brain regions post-partum (day 3 and 7) may explain why progesterone no longer inhibits maternal behaviour during the lactation period because there are fewer intracellular progesterone receptor for progesterone to act upon in brain regions critical for maternal behaviour expression [350].

Application of RU486, an intracellular progesterone receptor antagonist, prevents progesterone from inhibiting maternal behaviour development in estrogen and progesterone-treated pregnancy-terminated rats [350]. Systemic administration of RU486 to mice also caused mild to moderate disruption of maternal behaviour [350, 354]. Although these studies seem to only highlight the importance of the intracellular progesterone receptor as the mechanism by which progesterone manipulates maternal behaviour, there is evidence that this is not the whole story.

RU486 application in estrogen only treated pregnancy-terminated rats had no effect on maternal behaviour onset latency [350]. This could be a RU486 dosage issue but this seems unlikely as doses lower than 5mg (used in this study) are effective in terminating pregnancy indicating progesterone may act through another mechanism other than via intracellular progesterone receptors to inhibit maternal behaviour [350]. RU486 also affects glucocorticoid receptors, so to be certain that the effects are via the progesterone receptor, experimentation with ZK28299; an antagonist specific only to intracellular progesterone receptor is warranted [350].

In the MPOA between 50 and 95% of its cells synthesize GABA [352]. During maternal behaviour more than 50% of GAD containing cells in the MPOA exhibited Fos suggesting GABA functioning may be important in this region for maternal behaviour control [355]. This may indicate the involvement of allopregnanolone (AP), the progesterone metabolite, as one of its most potent functions is to facilitate the inhibitory action of GABA acting via the $GABA_A$ receptor [356-358]. It was observed that the actions of progesterone in preventing early birth may be mediated by AP as the SON OXT neurones are influenced by the inhibitory actions of GABA controlled allosterically by AP [352]. It is important to note that the MPOA is not the only brain area where progesterone may act to inhibit maternal behaviour, as progesterone implants alone in the MPOA were ineffective in preventing maternal behaviour, thus progesterone may act at multiple sites to inhibit maternal behaviour [347, 350, 352]. For example, it is suggested that progesterone may inhibit maternal behaviour by decreasing MPOA functioning and increasing VMN functioning [350]. Also, maintaining high levels of progesterone in pregnancy-terminated rats also results in lower Fos expression in the MPOA, LS and BnST

indicating progesterone may prevent maternal behaviour by inhibiting neural activity in the all or one of these regions [347]. However, it is important to note that these changes in maternal behaviour or Fos expression only occur in rats who express maternal behaviour, therefore the lack of pup contact, meaning no olfactory and sensory input, in non-maternal mothers could be the cause of lower maternal behaviour or Fos expression [347].

1.6.2 Allopregnanolone in the brain

AP is derived from peripherally sourced progesterone or cholesterol through the actions of the enzymes, 5α-reductase and 3α-hydroxysteroid dehydrogenase, which are available extensively throughout the brain (see Fig. 1.4) [359-361]. AP is described as a neurosteroid due to its ability to be allosterically modulate the $GABA_A$ receptor in the presence of GABA resulting in manipulation of many behavioural and physiological functions [362]. AP has little or no affinity for the intracellular progesterone receptor, therefore any impact AP has on behavioural or physiological outputs are due to modulation of $GABA_A$ receptor functioning which causes increases in the Cl^- influx, channel opening and efficacy of other positive $GABA_A$ receptor modulators e.g. benzodiazepines [342, 351, 359, 363, 364]. The enzymes, 5α-reductase and 3α hydroxysteroid dehydrogenase, responsible for AP production are generally found in the primary output neurones and glia in the brain, therefore AP may impact upon the $GABA_A$ receptor in one of three main ways (1) AP may be released by the cell to act in a paracrine fashion on a distal cell; (2) AP may act on the $GABA_A$ receptors of the same neuron in which it was synthesised after being released i.e. an autocrine fashion; (3) AP may work on intracellular sites of the $GABA_A$ receptor in the cell it was synthesised in, accessed by lateral diffusion

through the plasma membrane [359]. Research is linking changes in AP levels with many emotional disorders possibly due to AP causing dysfunction in GABA neurotransmission [342, 365, 366].

1.6.3 Progesterone, allopregnanolone and anxiety

Maternal aggression is closely linked with fear and anxiety and progesterone is known to be anxiolytic in rodents [15, 27, 203, 237-240]. Furthermore, it was uncovered that these anxiolytic actions of progesterone are mainly due to its conversion to the metabolite, AP [27]. ICV AP administration to proestrous rats resulted in lower anxiety-like behaviour display on the EPM than vehicle-treated proestrous rats [367]. This effect could be blocked by picrotoxin, a GABA-gated Cl^- blocker [367]. Rats in proestrous display less anxiety behaviour in the open field test

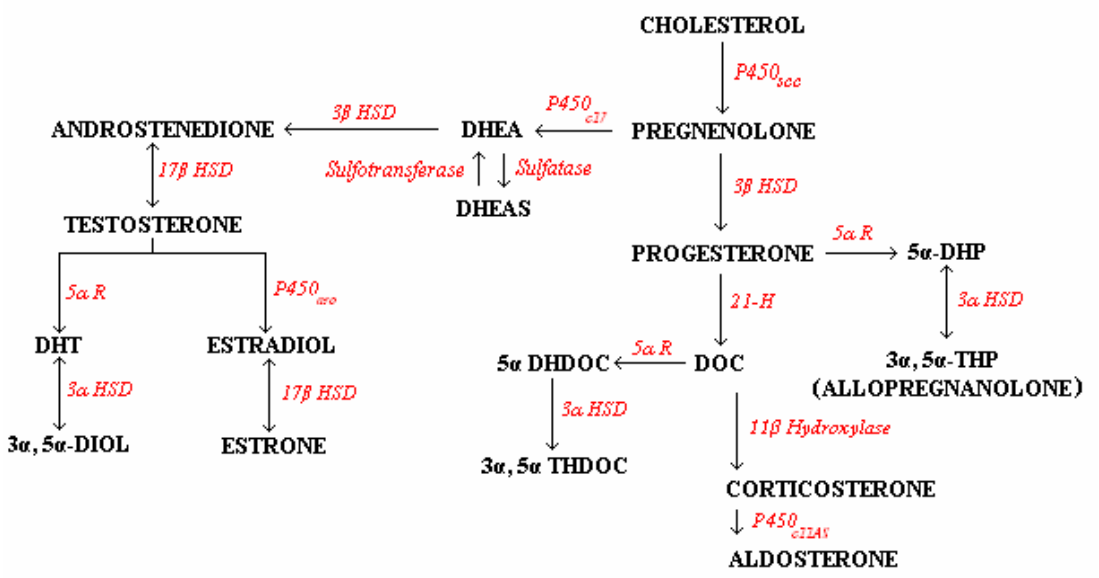


Figure 1.4: Neurosteroidogenesis pathways in the brain. Modified from Stoffel-Wagner (2003) and Mellon et al (2002). P450scc, scc=side chain cleavage, P450c11as = mitochondrial aldosterone synthase, 3β-HSD = 3β Hydroxysteroid dehydrogenase, 17β-HSD = 17β hydroxysteroid dehydrogenase, 5α-R = 5α-reductase, 3α-HSD = 3α hydroxysteroid dehydrogenase, 21-H = 21 hydroxylase, DHEA = dehydroepiandrosterone, DHEAS = dehydroepiandrosterone sulphate, 5α-DHP = 5α-dihydroprogesterone, 3α, 5α-THP = 3α, 5α-tetrahydroprogesterone, DOC = deoxycorticosterone, 5α DHDOC = 5α dihydrodeoxycorticosterone, 3α, 5α THDOC = 3α, 5α tetrahydrodeoxycorticosterone, DHT = dihydrotestosterone.

and forced swim test than diestrous or male rats normally; this could be due to the higher levels of AP in the hippocampus normally seen during proestrous [368]. Furthermore intrahippocampal or systemic finasteride administration, a 5α reductase inhibitor, to reduce hippocampal AP levels in proestrous rats prevented lower anxiety behaviour profile in the open field and forced swim test [368]. In ovariectomised rats, lower anxiety behaviour was displayed on the EPM following systemic progesterone administration in conjunction with enhanced Cl^- flux mediated by GABA [369]. Systemic progesterone application also caused an increase in AP levels in the cortex and blood serum of the ovariectomised rats indicating that the anxiolytic effect of the progesterone administration are actually mediated by its metabolite, AP [369]. These anxiolytic effects of progesterone and AP are not restricted to cycling females.

Progesterone withdrawal in pseudopregnant rats by ovariectomy increased in anxiety exhibited on the EPM compared to control and pseudopregnant rats [370]. Administration of a 5α reductase (MK-906) inhibitor to a pseudopregnant rat causes an increase in anxiety on the EPM [370]. This increased anxiety was linked with a decrease in $GABA_A$ receptor functioning which could be as a result of an observed increase in the α4 subunit of $GABA_A$ receptor; however this increase was only examined in the hippocampus [370]. Thus if the dramatic changes in AP and progesterone levels around parturition are not able to be normalised then this could result in increased anxiety, a major symptoms of postnatal depression and postmenopausal dysphoria disorders; but also provides an excellent model of how to study this further as the progesterone that the pseudopregnant rats experience is from an endogenous source [370]. In males, treatment with progesterone or pregnanolone

increased time in the open arms of the EPM, decreased anxiety behaviour in a defensive burying paradigm, and prevented inducement of anxiogenic behaviour on the EPM by an inescapable electric shock [371, 372]. Pregnanolone also works in the brain by positively modulating the $GABA_A$ receptor, showing that in males and female rats it is the ring A reduced metabolites (see Fig. 1.5), AP and pregnanolone, which mediate the anxiolytic effects of progesterone [371].

New research is beginning to focus on how AP is involved in modulating the effects of progesterone on maternal behaviour including maternal aggression during the peri-partum period; the time when a mother's emotional state is not only important for her ability to care for her offspring but also the offspring's future mental well being [14-17].

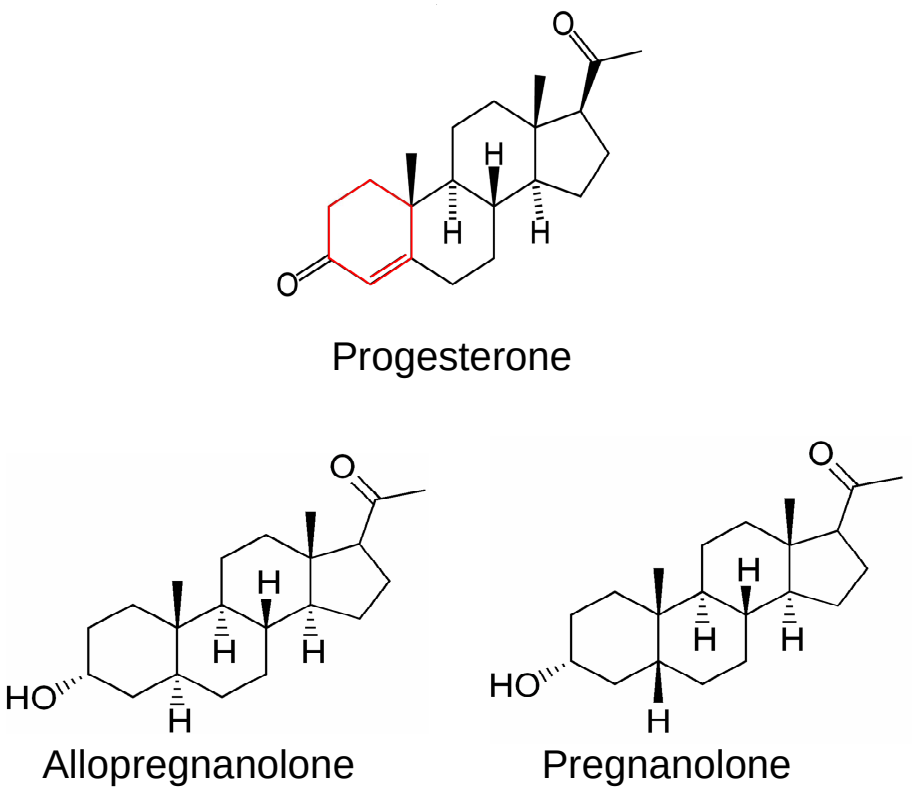


Figure 1.5: Structure of progesterone and its ring A metabolites. Diagram depicts the structure of progesterone. The ring A structure is highlighted in red. The reduction of this structure forms pregnanolone and allopregnanolone which are proposed to be the structures that mediate the anxiolytic actions of progesterone.

1.7 GABA

GABA is the main inhibitory neurotransmitter in the brain where it appears in roughly one third of neurones [373-375]. GABA is synthesised from glutamate, the major excitatory neurotransmitter, by the rate limiting enzyme, glutamic acid decarboxylase (GAD) of which there are two isoforms GAD 65 and 67 [375]. GABA has two types of receptor it acts upon to cause a change in ion flow and thus cell activity, the ligand gated ion channel $GABA_A$ and the G-protein coupled $GABA_B$ receptors [376]. The $GABA_A$ receptor works to manipulate the flow of chloride ions across the cell membrane; during development GABA acts in an excitatory manner but its action changes to inhibitory in adulthood [377]. The actions of GABA on its $GABA_A$ receptor will be the focus in this thesis because of the ability of AP to modulate $GABA_A$ receptor functioning to impact upon anxiety and aggression as discussed above [361]. $GABA_A$ receptors are also well established in mediating aggressive behaviour in rodents, especially heightened male aggression [342, 378-380]. Finally, recent research has linked changes in $GABA_A$ receptor functioning facilitated by progesterone or its metabolite AP in premenstrual tension and other disorders connected to the menstrual cycle or the peri-partum period [361, 381].

1.7.1 GABA and aggression

Female aggression has many behavioural similarities to the different forms of male aggression, hence neurotransmitters controlling linked with male aggression may also play the same role in regulating maternal aggression [378]. There is substantial evidence for GABA neurotransmission in the control of male aggression [342, 373]. Direct manipulations of GABA receptors by injecting GABA agonist inhibit aggression in male mice [342, 373, 382, 383]. Low levels of GABA correlate with

higher aggressive behaviour in mice and hamsters [384-386]. Furthermore, male GABA transporter subtype 1 (which controls GABA reuptake) KO mice display reduced aggression compared to wild types, so prolonged GABA neurotransmission prevents aggressive behaviour [374]. However, it is also well established that positive $GABA_A$ receptor modulators, including ethanol, benzodiazepines and AP work to heighten aggressive behaviour displayed in males under a range of conditions [342, 378]. Furthermore, benzodiazepine antagonists, specific for the α1 subunit of the $GABA_A$ receptor, are able to prevent increases in aggressive behaviour which may explain why not all $GABA_A$ receptor positive modulators work to decrease aggression because they need to act on specific $GABA_A$ receptor subunits [378, 380]. Hence GABA under certain conditions can work to escalate aggression or prevent it; one possibility is that GABA acts differently on different types of aggressive behaviour, it may be stimulatory to offensive but inhibitory to defensive [342, 373, 378]. Only recently has GABA been investigated in relation to maternal aggression even though evidence links GABA neurotransmission with disorders of the estrous cycle and the peri-partum period [361, 370, 381, 387]. Inhibition of $GABA_A$ receptors within the LS resulted in significant reduction in maternal aggression expression in lactating mice [387]. Furthermore, GABA transmission has been implicated in the control of OXT release, which may modulate HPA axis activity during basal and stressful situations [107, 388]. Thus, evidence is starting to build that GABA neurotransmission may have an important role in the regulation of maternal aggression as well as in male aggression.

1.7.2 GABA transmission and anxiety

GABA neurotransmission in rodents has been observed to be linked with anxiety and depression behaviour [361, 375, 389]. Application of bicuculline, a $GABA_A$ receptor antagonist, to proestrous rats increases anxiety behaviour display on the EPM and open field test [389]. In GAD 65 KO mice, increasing GABA levels during development were delayed compared to wild type controls [375]. Furthermore they display a higher anxiety profile in the forced swim and light/dark tests and lower aggression in an intermale aggression test [375]. Hence GABA synthesis controlled by GAD65 is important for the control of emotional behaviour [375].

$GABA_A$ receptor activity links GABA transmission with premenstrual tension disorders and possibly postnatal depression [361, 381]. During the oestrous cycle of rats, there are changes in the $GABA_A$ receptor subunit composition that coincide with the rise and fall of progesterone levels [381]. Furthermore these subunit alterations cause different $GABA_A$ receptor functioning; for example in the PAG during late diestrous the change in $GABA_A$ receptor subunits results in reduced GABA activity and hence increased PAG activity, an area closely linked with emotional behaviour [42, 140, 381, 390]. Thus it is proposed that the plasticity of these changes within women may be the cause of some the symptoms of premenstrual tension [381]. This plasticity of the $GABA_A$ receptor has also been implicated in postnatal depression where lack of the $GABA_A$ receptor δ subunit throughout pregnancy in mice resulted in poor maternal care and depression behaviour expression post-partum [361]. In the heterozygous KO $GABA_A$ receptor δ subunit mouse, administration of a $GABA_A$ receptor agonist, THIP, alleviated poor maternal care to result in greater pup survival [361]. Thus, ability to regulate $GABA_A$ receptor expression and activity during the

peri-partum period is important to maintain normal behaviour expression and manipulation of $GABA_A$ receptor δ subunit activity or expression provides one model to study how post-partum depression may occur and a potential therapeutic target [361].

GABA neurotransmission therefore is not only important in the control of aggressive behaviour, especially in males, but also anxiety and depression which may be related to maternal aggression expression [361, 371, 375, 381, 389]. GABA neurotransmission is also closely correlated with many emotional disorders that could be mediated by changes in progesterone or its metabolite levels, which change dramatically during pregnancy and parturition [361, 381, 389]. Hence research is starting to focus on how GABA transmission may control behaviour during this period and indicate possible targets for drugs.

1.8 The aim of this thesis

The aim of this thesis is to understand and gain knowledge into the control and regulation of maternal behaviour, specifically one aspect; maternal aggression. Maternal aggression is the focus of this thesis because it is hard to induce aggression in non-maternal rats and maternal aggression is expressed specifically only during the post-partum period. Therefore there is a short time period where the behaviour is switched on and off. Thus manipulation of neuromodulators that may result in changes to maternal aggression that can be readily observed. During the peri-partum period when maternal aggression is expressed, there are numerous changes occurring in the female. Hormone levels are rising and falling, alongside changes in receptor expression and functioning to maintain normal behaviour expression. If these do not

occur normally, not only may they result in disorders in the mother but they can also have lifelong consequences for the offspring [14-17]. Hence the importance of understanding what changes are occurring and what part they play in the maternal behaviour circuitry that organises maternal aggression.

For studies in this thesis it was decided to explore the effects of OXT and AVP on maternal aggression because there is substantial evidence for these neuropeptides being involved in numerous social behaviours, including affiliation, social memory and aggression, all which are important during the post-partum period for offspring survival [3, 44, 227, 228, 290, 304, 307, 314, 316-319, 330, 333, 334, 339]. Also investigated were roles of GABA and AP, because AP can modulate GABA neurotransmission by potentiating the action of GABA at the $GABA_A$ receptor [342, 359, 362]. Furthermore this effect has been linked with emotional disorders especially those which are related to changes in progesterone levels [361, 370, 381]. During pregnancy progesterone levels, and consequently AP, change dramatically, so these changes may be linked pregnancy and post-partum disorders if GABA neurotransmission is dysregulated when the levels of AP and progesterone fall dramatically post partum.

Chapter Two: Materials and methods

2.1 Animals

Virgin female Sprague-Dawley rats (Charles River) weighing between 200 and 250g were used in all studies. The rats were group housed (n = 5/cage) prior to experimental setup and fed on normal rat chow diet (RM1). One week prior to mating rats were moved onto breeding rat chow diet (Teklad 2019) and housed overnight with a male until the presence of a seminal plug (Pregnancy day 1) was detected. Once pregnant, rats were caged individually for the duration of the experiment. Rats were housed under standard laboratory conditions (12h light/dark cycle: lights on at 7:00, off at 19:00; temp (19-21^{o}C); humidity (50-55%) and ad libitum access to food and water). Virgin females rats were group housed (n = 5/cage) for the length of the experiment under standard laboratory conditions as detailed above. Animal care and use protocols were approved by the University of Edinburgh and under UK Home Office Licence guidelines and regulations.

2.2 Injections and sampling

2.2.1 Subcutaneous (s.c.)

Subcutaneous injections were either performed at the base of the neck or on the back close to the hind legs. An injection was made into the centre of a fold of lightly pinched skin.

2.2.2 Intraperitoneal (i.p.)

The rat was held on its back and the skin around the stomach area was gently lifted and the needle inserted into the body cavity. The needle was pulled back slightly before injection.

2.2.3 Vaginal smears

To collect vaginal cells, 1-2ml of physiological saline (0.9% w/vol) was gently flushed in and out of the vagina using a plastic pipette. The saline containing the cells was expelled onto a glass microscope slide and left to air dry. Once the smear was dry, cells were fixed in acetic alcohol fixative (AAF) for about 10 sec, drained and dipped into toluidine blue (1%; ~10 sec) drained and coverslipped with glass coverslips (VWR) using DPX mountant (BDH, Poole).

Cell morphology was examined under a light microscope with a 20X objective mountant to determine the phase of the oestrous cycle (see table 2.1 for diagrams and descriptions of cycle phases).

2.2.4 Blood sampling using tail tipping

The rat was gently restrained by being wrapped in a towel with the tail was exposed. A small nick was then made about 2.5cm from the tip of the tail using a sterile surgical blade (Swann Morton). The tail was then gently massaged to encourage blood flow. The blood was collected in heparinised capillary tubes (Hawksley, Sussex), transferred to sterile Eppendorf tubes which were spun in a centrifuge at 2000xg for 3-5 min. The plasma was decanted by pipette into a fresh labelled sterile Eppendorf tube and stored at -20^{o}C until radioimmunoassay for levels of circulating progesterone.

2.3 Surgery

2.3.1 Intracerebroventricular (ICV) cannulation

SD rats on day 2 of lactation, weighing 276±7.6g (number of rats = 23), were anaesthetised with isoflurane (Dunlop; 0.5-1L/min) mixed with nitrous oxide (2L/min) and oxygen (2L/min). Fur was shaved from the top of the head with

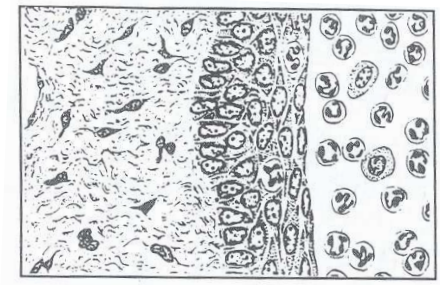
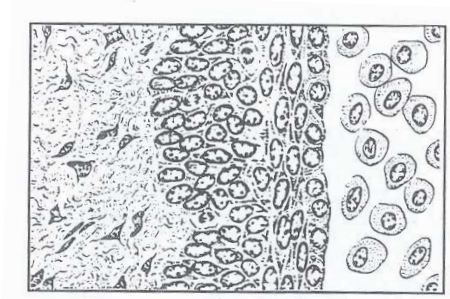
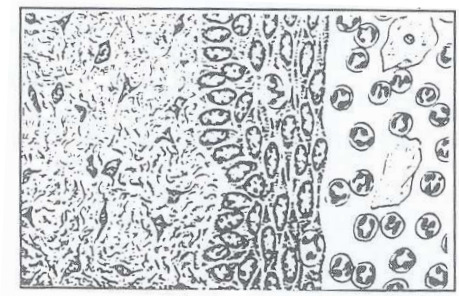
Phase of Cycle	Diagram	Description of Cell Morphology
Diestrous		Mainly leucocytes with the possibility of one or two nucleated (intermediate) epithelial cells. Plasma levels: Estrogen 1.5ng/ml Progesterone 30ng/ml Typically 57h
Proestrous		Masses of intermediate nucleated epithelial cells. Plasma levels: Estrogen 4.5ng/ml Progesterone 80ng/ml Typically 12h
Estrous		Largely clumps of cornified epithelial cells (75%) with a few nucleated intermediate epithelial cells (25%). Plasma levels: Estrogen <1ng/ml Progesterone 20ng/ml Typically 12h
Metestrous		Chiefly polymorphonuclear leucocytes with a few cornified epithelial cells. Plasma levels: Estrogen <1ng/ml Progesterone 20ng/ml Typically 15h

Table 2.1: Vaginal smear cycle phase morphology. Modified from table 6-1 in The Laboratory Rat by Mark A. Suckow, Steven H. Weisbroth and Craig L. Franklin [391].

clippers. The rat was then placed into a stereotaxic frame, making sure the head was securely positioned by the ear bars. During this time, a nose cone supplied a constant flow of anaesthetic to the rat.

The rats head was sterilized with povidone-iodine antiseptic solution **(**Vetasept) and a 2cm midline incision made with a sterile surgical blade (Swann Morton). Blood was cleaned from the skull with sterile cotton wool buds. Bregma is the point where the sagittal suture crosses the coronal suture and is used a place of reference on the skull to position a hole for implanting cannula into specific brain regions [392]. Bregma is used as a point of reference because in all rats, from juvenile until mature, bregma is consistently positioned above the most forward crossing fibres of the anterior commisure [392]. Bregma was located and a hole was drilled above the right ventricle (1.6cm lateral to the midline, 0.6cm posterior to bregma) using a hand held electrical drill (Precision PCB/modal) with 2.3mm shaft drill bit (Fine Science Tools). Two further holes were drilled for screws to be implanted; one right rostral and one left caudal to the hole for the cannula. Two screws (1.6x3mm cheese head A2; Precision Technology Supplies Ltd) were inserted into the holes adjacent to the cannula and acrylic dental cement (Kemdent) was used to secure the cannula to the skull. Rats received 1ml of physiological saline (i.p.) to correct for dehydration during surgery and a 4mg/kg injection of Rimadyl (Carprofen; Dunlop) s.c. for pain relief prior to waking up. Rats were allowed to recover on a vet bed heating pad (Vet Tech Solutions Ltd).

2.3.2 Bilateral microdialysis using cannulation

The surgery was performed as above until the point of finding bregma. Polyethylene cannula tubing (outer diameter 0.97mm, inner diameter 0.58mm; Plastics One) about

5cm long was attached to the outflow end of the microdialysis probe (3mm membrane length u-shaped; University of Regensburg, Germany). The microdialysis probe was checked for any faults and leaks by slowly injecting Ringer's solution (composed of [in mM] 147.1Na^{+}, 2.25Ca^{2+}, 4K^{+} and 155.6Cl^{-} at pH 7.4; Fresenius, Bad Hamburg, Germany) before attaching it to a holder on the stereotaxic frame. The probe was positioned vertically in the holding frame and its tip located positioned over bregma before it was moved to coordinates for implantation into the designated specific brain region (from bregma PVN -1.6 anterior/posterior, ±1.8 lateral, +9.1 depth; BnST -0.7, ±1.5, +6.5; LS +0.2, ±2.3 +6.0; MeA -2.0, ±3.5, +9.0 and SON -0.6, ±1.8, +9.4). In order to avoid major blood vessels, injections into the PVN and LS required the probe to be inserted at an angle (PVN ^{o}10, LS ^{o}20). The first probe was inserted in the left hand side of the brain and fixed into place by attaching it to one the screws with dental cement (Kemdent) which was set hard by exposure to a bright light (KL 1500 Compact, Schott AG, Mainz, Germany). This process was repeated for the second probe which was inserted into the right hand side of the brain. Again this probe was fixed in place using dental cement (Kemdent) to attach it firmly to the second screw and the skull. Once the cement was dry, cannula tubing 6cm long was attached to the inflow end of each probe. Ringer's solution (0.5ml) was flushed through gently to make certain the probe was still patent and intact. Dental cement was then used to firmly attach the tubing to the probes. Coloured tape was attached to the inflow (black tape) and outflow (white tape) tubing of each probe to keep them together and provide a means of identification of the direction of flow. Rats were injected s.c. with 0.03ml of antibiotic (enrofloxacin 3 mg/0.03 mL; Baytril, Bayer, Germany) and left to recover in their home cage for one day.

2.3.3 Microdialysis Sampling

At 08.00h on the day of behavioural testing, a syringe filled with sterile Ringer's solution, mounted on a microinfusion pump, was connected with polyethylene tubing (30-40cm long) to the inflow tubing of each individual microdialysis probe. To the outflow tubing a 1.5ml Eppendorf was attached via a 5cm length of polyethylene tubing for sample collection. For 2h prior to the first sample collection, the microdialysis probes were perfused with Ringer's solution at a rate of 3.3µl/min. Five successive 30 min samples then were collected from each rat. The first two samples were taken under basal conditions i.e. the rat was left undisturbed in her home cage. Sample 3 was collected during a 10 min maternal aggression test (as described below). Samples 4 and 5 were taken following the aggression test with the rat left undisturbed. The microdialysates were immediately frozen on dry ice and stored at -20^{o}C until they were analysed for levels of OXT or AVP by radioimmunoassay at the Max Plank Institute, Germany, by Prof Rainer Landgraf.

2.4 Behavioural Testing

2.4.1 Sensitization of virgin female rats to pups

On day one of pup-sensitization, 3 pups (aged between 2 and 8 days) were placed into the corner opposite the sleeping nest of the virgin female in her home cage. For the first 15 min, the rats were observed and any behaviour directed towards the pups (sniffing, licking, picking up or retrieving pups) was noted. Spot checks were then made at 30, 45 and 60 min after the start of the test with the position of the virgin and pups recorded each time. At the end of the observation (60 min), the pups remained with the virgin for 24h after which they were removed and 15 min later replaced with

3 recently fed pups from a different litter. This protocol was repeated daily until the virgin rat displayed full maternal behaviour or 13 days had passed. Rats were defined as being fully maternal (i.e. pup-sensitized) when they sniffed, licked and retrieved all 3 pups back to the nest within 15 min of the start of the observation test.

2.4.2 Maternal aggression test

A novel virgin female intruder (weighing between 200-250g which has been housed in a different room) was marked with black pen on its back to allow for identification and placed into the home cage of a lactating rat in the presence of her pups (8-18) for a specific period of time (either 10 or 30 min). Behaviour was recorded via a digital camcorder and later analysed for defensive, offensive and maternal behaviours using Noldus Observer Video Pro (see appendix 3 and 4 for more detail). Following 30 min exposure to the resident, the female intruder was removed and returned to her home cage. No female intruder was used more than once per day.

2.4.3 Maternal behaviour test

The behaviour of lactating rats in their home cage with their pups present (8-18) was digitally recorded for 30 min.

2.4.4 Pup retrieval task

Prior to behavioural testing, the entire litter was removed from a lactating rat and the pups were covered in bedding to keep them warm in a separate cage out of earshot of the lactating rat. After 1h of separation, eight of the litter were randomly scattered around the home cage. Behaviour was digitally recorded until all eight pups were retrieved and returned to the nest or after 30 min. The rest of the litter was then returned to the dam in the home cage. The digital recording was analysed for latency to approach the pups or sniff the first pup, latency to retrieve the first pup back to the

nest, latency to retrieve the first four pups to the nest and latency to retrieve all eight pups to the nest.

2.4.5 Elevated plus maze test

An EPM consists of four horizontal arms coming out at 90^{o} to each other from a central area at the 48.5cm from the floor. Two of the arms are enclosed by walls (at a height of 38cm and length 127cm; closed arms) and two arms have no walls (length 127cm; open arms). Rats were placed into the centre section of the four arms of the EPM and allowed to move freely whilst being digitally recorded from above for 5 min. The behavioural recording was later analysed using Noldus Ethovision for latency to enter open/closed arms, time spent in each arm and total distance moved (see appendix 4 for more detail).

2.4.6 Behavioural analysis

The behavioural videos were uploaded onto Noldus Observer Video Pro Version 5 or XT 7.0 for maternal aggression or maternal behaviour analysis or Ethovision for EPM test analysis. Appendix 4 shows the graphical user interface for each program. The behavioural videos of the females were analysed for all behaviours. For a maternal aggression test, offensive (latency to attack, number of attacks, biting, rearing, sniffing), defensive (immobility), normal maternal behaviours (grooming/licking, pup carrying, nursing) and normal behaviours (eating, drinking, exploring; see appendix 3) were all examined.

2.5 Transcardial Perfusion for Fixation

The rat was given a lethal overdose of anaesthetic (2.8ml/kg sodium pentobarbital; i.p.) 90 min after the start of behavioural testing and perfused via the left ventricle

with heparinised saline (250ml) and then fixed with 4 % paraformaldehyde (300ml) using a peristaltic pump. The brains were carefully removed from the skull using scissors and bone rongeurs, put into a 4% paraformaldehyde and 15% sucrose solution and stored at 4-5^{o}C until the brain had sunk (usually overnight). The brain was then placed in a 30% sucrose in 0.1M phosphate buffer at 4-5^{o}C for 1-2 days. Once sunk in this solution, the brain was removed, the excess solution dried off with a paper towel, and divided by coronal cuts with a blade (Fischer Scientific) into thick slices including the OBs (cut just prior to the lateral septum), main brain (preoptic regions and hypothalamus) and hind brain (cut just into the cerebellum) sections. Each section was frozen on foil on dry ice and then wrapped in foil before being placing into labelled plastic bags and stored at -70 ^{o}C.

2.6 Immunocytochemistry

2.6.1 General principles

Immunocytochemistry (ICC) is a sensitive and adaptable method for ascertaining the specific molecular components of a tissue or cell via an antigen-antibody reaction marked by a visible tag [393, 394]. There are several methods for detection varying in speed and sensitivity. The method used in this thesis was the Avidin-Biotin Complex (ABC) method which has excellent sensitivity and specificity and the process can be performed quickly. The ABC method involves application of an unlabelled primary antibody specific to the molecule intended for detection. Next, a biotinylated secondary antibody raised against the primary is used. Thirdly the avidin-biotin complex, where avidin has bound to a biotinylated peroxidase due its extremely high affinity for biotin, is applied (Figure 2.1). This is then visualised

using diaminobenzidine tetrachloride (DAB), a substrate of peroxidase, forming a visual brown tag (with the addition of nickel ammonium sulphate the reaction product turns black) enabling visualisation of the labelled molecule under a light microscope and allowing quantifiable analysis.

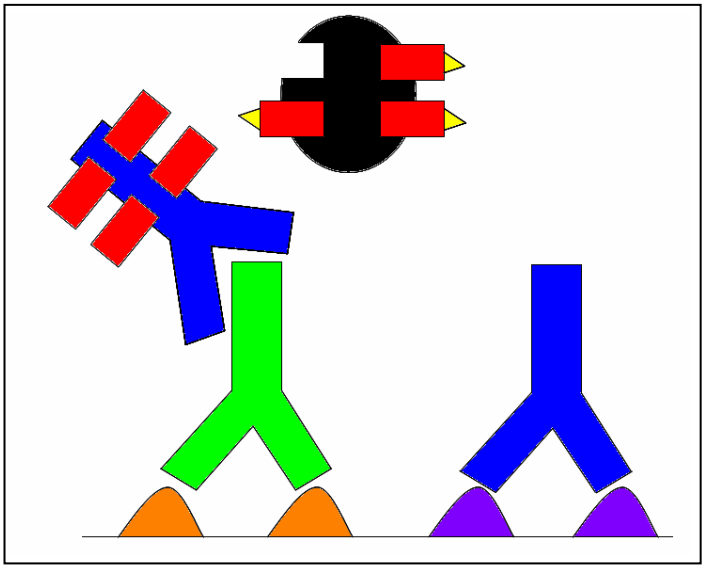


Figure 2.1: Avidin-Biotin complex method for immunocytochemistry. Immunocytochemistry is used to identify molecules in tissues by an antigen-antibody binding mechanism. In this figure, the primary antibody (green) binds to the molecule within the tissue. A biotinylated secondary antibody (blue and red) is applied and binds to the primary. Finally the avidin-biotin complex is used consisting of avidin bound to a biotinylated peroxidase (black, red and yellow). The tissue can then be washed with a substrate of peroxidase such as diaminebenzidine tetrachoride which provides a visual tag for the molecule. Non-specific binding blocked by using normal serum (blue) during the procedure. Modified from Figure 2.3, Immunocytochemistry Practical Applications in Pathology and Biology, Polak and Van Noorden (1983).

2.6.2 Gelatinization of slides

Slides were slowly dipped (to avoid air bubbles) 2 to 3 times in the filtered slide gelatinization solution and then left to dry overnight at room temperature (appendix 5).

2.6.3 Immunocytochemistry for the immediate early gene (c-fos) Fos protein

Brains were sectioned at 52 μm on a freezing microtome and sections were collected into 0.1M PB. Sections were then washed in 0.1M PB with 0.2% Triton X-100 (4x15min, PB-T) and once in 0.1M PB (5 min). Endogenous peroxidase was blocked

with 0.3% H_2O_2 in 0.1M PB for 15 min following which sections were rinsed with 0.1M PB-T (3x10min). Non-specific immunoreactivity was blocked by incubating sections with 1% normal sheep serum in PB-T for 30 min. Sections were then incubated with Ab-2 Fos antibody (1:1000, Rabbit Polyclonal; Calbiochem, UK) in 1% normal sheep serum in PB-T for 48h in sealed labelled vials at 4^{o}C during which sections were gently agitated daily.

After 48h, sections were washed in 0.1M PB-T (3x10min) before being incubated in biotinylated anti-rabbit Ig (1:1000) and normal sheep serum (1:100) in 0.1M PB-T (Vectastain ABC Elite Kit PK6100; Vector Labs) for 1h. Sections were washed in 0.1M PB-T (3x10min) before incubation for 1h in the Avidin DH and biotinylated horseradish peroxidase complex (both 1:500 in 0.1M PB-T; Vectastain ABC Elite Kit PK6100; Vector Labs). Sections were then rinsed with 0.1M PB-T (2x10min) and 0.1M sodium acetate (5min) before visualisation with DAB (0.25mg/ml) and nickel sulphate (0.025g/ml) with 0.3% H_2O_2 for up to 10 min. This reaction was terminated by immersion in 0.1M sodium acetate and then 5 washes of 0.1M PB. Sections were then serially mounted onto gelatinized microscope slides (Menzel-Glaser) and left to dry overnight before going through a series of increasingly concentrated ethanol solutions (70%, 90%, 95%, 100% and 100%; each for 5 min). Sections were then cleared in xylene (2x5min) before being coverslipped with glass coverslips (25mmx60mm; VWR) using DPX mountant (BDH, Poole).

2.6.4 Double labelling immunocytochemistry

2.6.4.1 Oxytocin receptor (OTR)

Rats whose brains were to be processed for OTR ICC were perfused with the OTR fixative (see appendix 6 for recipe). Once the brains were collected, they were

sectioned coronally and processed for Fos ICC as described above. Following visualisation, sections were washed with 0.1M PB (2x5min) and then 0.1M PB-T (2x5min). Any unreacted peroxidase was blocked with 0.3% H_2O_2 for 15 min and then by washing in 0.1M PB-T (2x10min) before being incubated in the OTR antibody (1:1000, rabbit polyclonal; kindly donated by Dr Fred Van Leeuwen from Maastricht University, Netherlands) for 48h in labelled vials at 4^{o}C. Sections were then rinsed in 0.1M PB-T (3x10min) before being incubated in biotinylated anti-rabbit Ig (1:1000) and normal goat serum (1:100) in 0.1M PB-T for 1h. Sections were washed again in 0.1M PB-T (3x10min) before incubation for 1h in the Avidin DH and biotinylated horseradish peroxidase complex (both 1:500 in 0.1M PB-T; Vectastain ABC Elite Kit PK6100; Vector Labs, UK). Sections were rinsed with 0.1M PB-T (2x10min) and the cytoplasmic OTR was visualised with DAB without nickel for 7-10 min resulting in a brown reaction product. This reaction was then terminated with several washes in 0.1M PB. Sections were mounted and coverslipped as previously described above.

2.6.4.2 Double labelling for Fos and glutamic acid decarboxylase (GAD) 65/67

The double labelling ICC method was followed as described above for OTR but using standard 4% paraformaldehyde fixative and rabbit anti-GAD 65/67 polyclonal antibody (Chemicon International; 1:2000 dilution).

2.6.5 Methyl green counterstain

To record the location of cannula or microdialysis probe placement in the brain, coronal sections were cut at 52 μm on a freezing microtome and mounted onto gelatinized slides. They were then rinsed in distilled water and methyl green solution (0.5% methyl green in 0.1M sodium acetate, pH 4.2; Sigma) was applied. Slides

were left at room temperature for 5 min before being rinsed with distilled water and then dipped 10 times in 95% ethanol followed by 100% ethanol (twice). Finally, they were cleared in xylene (5 min) before being coverslipped as described above using DPX mountant (BDH, Poole).

2.6.6 Quantification technique

Slides were coded so the experimenter was blind to which treatment group they belonged to and examined under a light microscope (objective 20X or 40X). For Fos ICC only, cells with black or dark grey nuclei (see Fig. 2.2a for examples) were counted in the specific brain area of interest defined using The Rat Brain in Stereotaxic Coordinates by G. Paxinos and C. Watson (see appendix 2 for more detail on brain areas examined) [392]. For double ICC, only cells with a clear dark brown nucleus and brown cytoplasmic staining were counted (Fig. 2.2b).

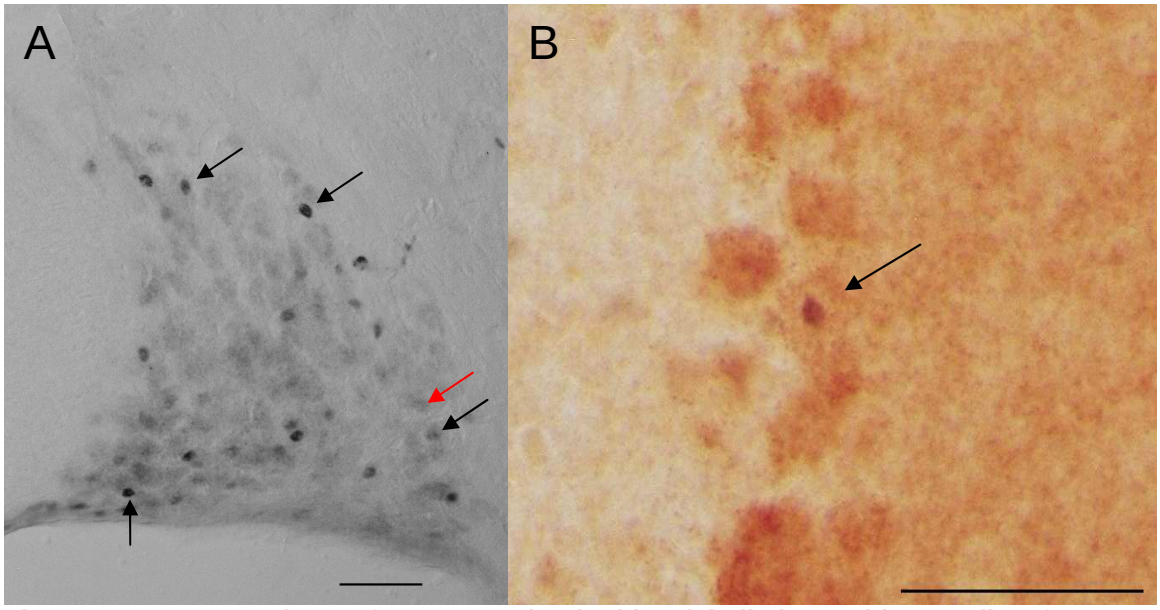


Figure 2.2: Examples of Fos and double labelled positive cells from immunocytochemistry. Fos positive cells (black arrows; A) and a Fos positive cell too pale to be included in the count (red arrow; A) in the supraoptic nucleus of an aggression tested rat. A cell was only counted as double labelled if it had a distinctive dark brown nucleus surrounded by a paler brown but clearly stained cytoplasm as shown in figure B which is an example of double labelled Fos and oxytocin receptor cell (black arrow; B) in the olfactory bulb of an aggression tested rat. Scale bar = 50μm.

2.7 In Situ Hybridisation

2.7.1 General principles

In situ hybridisation (ISH) is an extremely sensitive method enabling detection of specific mRNA sequences in certain tissue or cell groups [395, 396]. The method uses a probe, made from radioactively labelled single strands of DNA or RNA complementary to the specific cellular mRNA, to hybridise to mRNA strand of interest under particular conditions to form a stable complex. The radioactive signal is then detected by autoradiography where liquid emulsion or autoradiographic film is applied to the slides and then left for a defined exposure time before developing. The probe used here was a 396 bp riboprobe complementary to the sequence encoding the fifth to the midseventh transmembrane regions of the V1a receptor kindly donated by Dr Stephen J. Lolait from the University of Bristol.

2.7.2 Brain sectioning

Brains were serially sectioned at 15 μm using a cryostat and were collected onto labelled polysine microscope slides (4 sections per slide; VWR). Six polysine slides were cut as a series, with every 7^{th} section being collected onto a gelatinized microscope slides (Menzel-Glaser) to act as a marker slide. Once four sections had been collected onto each slide, the 6 polysine slides in labelled order were put into a slide box with a silica gel bag and kept at $-25^{o}C$. Once the box was full, the slide box was sealed with tape and stored at $-80^{o}C$ with the silica gel bag still inside to reduce condensation ice damage to the tissue at this temperature. The 7^{th} slide in each series, or marker slides, was dipped into AAF fixative for 10 sec, the excess drained off and then dipped into 1% methyl violet. The excess was washed off under running water and the slide left to dry. The marker slides were stored at room temperature and used

to identify the location of each brain section with reference to the Rat Brain in Stereotaxic Coordinates by G. Paxinos and C. Watson, so that slides with brain regions of interest could be removed from storage as necessary.

2.7.3 Plasmid linearization

On ice, the anti-sense (signal), sense (control) and double digest template construction solutions were made up in Eppendorf tubes according to the amounts listed below:

Anti-Sense (100µl digest)	Sense (50µl digest)	Double Digest (25µl digest)
20µl DNA	20µl DNA	5µl DNA
4µl Anti-sense Restriction Enzyme	4µl Sense Restriction Enzyme	2µl Anti-sense Restriction Enzyme
		2µl Sense Restriction Enzyme
10µl Buffer	10µl Buffer	10µl Buffer
66µl DEPC-H_20	66µl DEPC-H_20	33µl DEPC-H_20

These were then vortexed and incubated in a 37^oC water bath for 2h (they were vortexed again after 1h in a water bath). Following incubation, 100µl of phenol:chloroform isoamyl alcohol (Sigma) was added, the Eppendorf tube was then vortexed and centrifuged for 5 min at 16250xg. The top layer was decanted into a fresh Eppendorf tube and an equal volume of chloroform:isoamyl alcohol (Sigma) was added. The plasmid was vortexed and centrifuged for 5 min at 16250xg again. The top layer was removed to a fresh Eppendorf to which 1/10 of its volume 5M NaCl (Sigma) and 2x the total volume (i.e. the volume of NaCl and the DNA containing solution) of absolute ethanol was added. The plasmid was then frozen on dry ice for 10 min, defrosted and centrifuged for 15 min at 16250xg. The supernatant was carefully decanted, taking care not to include the DNA pellet, and discarded. The DNA pellet was left to air dry for at least 60 min. Once dry the DNA pellet was

resuspended in TE buffer (15µl for anti-sense and 8µl for sense) and then stored at -20^{o}C.

2.7.4 Gel electrophoresis of linearised plasmid fragments

Gel electrophoresis was used to check correct linearization of the plasmid. The gel separates the plasmid fragments according to size; the shorter (in base pair) the length of the fragment, the further it will move through the gel. A DNA ladder was also included so that a scale of known lengths from which the length of the anti-sense, sense and double digest templates could be approximated and checked against expected values.

1g of agarose was added to 100ml of 1XTBE and dissolved gently. 10µl of DNA safe stain (Cybersafe) was added and the solution poured into the tray with comb in place making sure there were no bubbles. The gel was then left to set for about 60 min. Once set, the gel was then placed into the chamber and enough 1X TBE was added to completely cover the gel (by at least 1 mm). On ice, 2µl loading buffer and 8µl DEPC-H_2O was mixed with 2µl of the antisense or sense template. For the double digest, 5µl was added to 1µl loading buffer and 4µl DEPC-H_2O. The solutions were then added to the wells as laid out below:

DNA Ladder (10µl)	/ Anti-sense / Template (10µl)	/ Sense / Template (10µl)	/ Double Digest / (10µl)	/ DNA ladder / (5µl)

The voltage was set at 120V and the gel set to run for 60 min. Once run, the gel was imaged using Genescan and GeneSnap Program. The plasmid was expected to be about 400 bp long.

2.7.5 Radioactively labelling the linearised plasmid

To sterile Eppendorfs the following were added:

4µl 5X transcription buffer

2µl ATP
2µl GTP
2µl CTP
1µl 100mM DTT
2µl template (anti-sense or sense)
0.8µl RNase, ribonuclease inhibitor
8µl S^{35}-UTP (for sense only 4µl)
2µl RNase polymerase (SP6 for anti-sense, T7 for sense)

These were then incubated in water baths (40^{o}C for antisense and 37^{o}C for sense) for 2h. After 2h, 2µl of DNase was added to digest the template and the Eppendorfs vortexed before being returned to water baths at the same temperatures as above for a further 15 min.

2.7.6 Purification of labelled probe

The anti-sense and sense labelled probes were purified using NICK columns (Sephadex). The solution at the top of the NICK column was discarded and 1 ml of TE buffer added, shaken and also discarded. 3ml of TE buffer was then added to the NICK column and allowed to completely elute into the waste pot. The probe was then applied to the top of the column followed by 400µl TE buffer and left to elute entirely. Another 400µl TE buffer was added to the column and once the first drop had been discarded, the probe was collected into a fresh sterile labelled Eppendorf. 2µl of each eluted probe was pipetted into individual scintillation vials with 3.5ml of scintillation fluid and then counted using a Beckman beta counter. This was to check two things, firstly that the probe was radioactively labelled and secondly to get a value of probe activity (i.e. counts per min per µl of probe) required for the calculations in section *2.7.9.1.* If not used immediately, the probe was stored at -20^{o}C.

2.7.7 Tissue preparation and fixation

Slides with the section of interest, taken directly from storage at -70^{o}C, were put into racks and placed into 4% paraformaldehyde in PBS for 10 min and rinsed in 2 washes of 1X PBS (each for 5 min). They were then incubated in 300ml triethanolamine (Sigma) with 0.75ml acetic anhydride (Sigma) for 10 min to reduce background. Slides were rinsed again with 2 washes of 1X PBS (each for 3 min) before being dehydrated through increasing concentrations of ethanol (70%, 80% and 95%) for 2 min each and left to air dry for about 30 min.

2.7.8 Pre-hybridisation

In a humidifying box, blotting paper (2 layers thick) was placed to fit the bottom of the box and covered with 20ml of box buffer (4ml 20X SSC, 6ml DEPC- H_2O and 10ml deionised formamide). To each slide, 200µl of prehybridisation solution (50% prehybridisation solution with 50% deionised formamide) was applied and gently spread over. Slides were then laid in the humidifying box on a glass or perspex plate, ensuring that they were level, and incubated for 2h at 50^{o}C.

2.7.9 Hybridisation

The volumes required for the hybridisation solution were calculated using the formulae below.

2.7.9.1 Formulae for Hybridisation Solution Calculation

Total Hybridisation Solution Volume	=	Volume of Hybridisation Solution required per slide (200µl) x Number of Slides
Volume of Deionised Formamide	=	50% of Total Hybridisation Solution Volume
Volume of DTT	=	10µl x Number of ml of Hybridisation Solution

Volume of Probe = $\frac{\text{Radioactivity required (Total Hybridisation Solution Volume x 10 x } 10^6 \text{ cpm/ml)}}{\text{Probe Activity}}$

Volume of Hybridisation Buffer = Total Hybridisation Solution Volume – (Volume of Probe + Volume of DTT + Volume of Deionised Formamide)

Once calculated the hybridisation buffer and deionised formamide (Sigma) were added together, vortexed and incubated at 70^{o}C for 10 min. The mixture was cooled on ice for 1 min and the DTT (Sigma) added before again vortexing the solution. Slides were removed from the oven and 200µl of hybridisation solution was applied to each after draining off as much as possible of the prehybridisation solution. Slides were then returned to the humidifying box as before and incubated at 55^{o}C for 16-18h.

2.7.10 Post hybridisation washes

Slides were removed from the oven, drained and placed into slide racks and taken through 3 washes of 2X SSC (each 5 min) at room temperature.

2.7.11 RNase incubation

In a RNase humidifying box, one layer of blotting paper was placed in the bottom and 20ml of box buffer added. Following washes, 200µl of RNase solution (1µl RNase/2ml box buffer) was applied to each slide. Slides were then incubated in the humidifying box for 60 min at 37^{o}C.

2.7.12 Post RNase treatment washes

Following incubation, slides were returned to racks and washed in 2X SCC at room temperature for 30 min. They were then rinsed in 0.1X SSC twice at 65^{o}C; the first being 90 min and the second 60 min with the solution being left at room temperature to cool. A fourth wash was for 60 min in 0.1X SSC in solution that had been left to

cool to room temperature. Slides were then put through a series of increasing concentrations of ethanol (50%, 70% and 90%) in 0.3M ammonium acetate (each for 2 min) and left to dry overnight.

2.7.13 Application of autoradiographic emulsion

In a dark room with the lights off and the red light on, a bottle of emulsion (Ilford) was placed in a black box in a 40^{o}C water bath and left for 2h to melt. Again with the lights off and the red light on, the emulsion was poured into a glass slide dipping container carefully to ensure that no air bubbles formed in the emulsion. The slides were then dipped and left to dry in a light proof box overnight. Once dry (again with lights off and the red light on), slides were transferred into a slide box with a silica bag to reduce moisture, sealed with tape, wrapped in foil and sealed in a black bag again with tape. They were then stored at 4^{o}C for the desired exposure time. Extra test slides were always included and developed at intervals to ensure optimum exposure time.

2.7.14 Development of autoradiographs

In the dark room with the lights off and red light on, slides were removed from the slide box and put into slide racks. Next the slides were dipped in fresh developer for 10 min (Kodak D19, 80g in 500ml water). Slides were then dipped in distilled water and then placed into fixer (2x10min, Ilford Hypam rapid, 100ml in 400ml water), followed by two 5 min rinses in distilled water. Slides were then processed for haematoxylin and eosin background tissue staining. They were put into Shandon Harris haematoxylin (acidified, 100%; Thermo Electron Corporation) for 5 min before being rinsed with water. Then slides were dipped twice in acid alcohol, rinsed with water again and left in Scott's tap water substitute (STWS; see appendix 5 for

details) for 3 min. Slides were then washed in water (running) for 3 min before being dipped into eosin solution (1%) for 3 min. They were rinsed with water dipped into potassium aluminium solution (5%; see appendix four for details) for 3 min. Finally slides were rinsed again in water before being taken through a series of increasing concentrations of ethanol (70%, 90%, 95%, 100% and 100% for 5 min each), cleared in xylene (two 5 min washes) and coverslipped (25x60mm, VWR) with DPX mountant (BDH Poole).

2.7.15 Quantification technique

Slides were coded so the experimenter was blind to treatment group. The silver grain density of neurons within the area of interest were quantified under a light microscope (objective 40X) aided by a computer image analysis system (Open Lab; Improvision Lexington MA). Three measurements were taken per area of interest using a fixed shape and three background measurements were collected from tissue close to the area of interest which exhibited little or no signal. Background measurements were then averaged for each rat and the average subtracted from each area of interest measurement for the same rat. The average silver grain density of the area of interest for each rat was then calculated.

2.8 Receptor Autoradiography

2.8.1 General principles

Whilst ISH and ICC provide detailed and important information about the cellular and tissue location of specific receptors or molecules, they cannot inform us about the functional binding affinity of these locations. Receptor autoradiography, on the other hand, has poor cellular resolution but can provide the binding site location of

specific receptors. Receptor autoradiography involves applying a radioactively ligand specific to the receptor of interest to fixed tissue section [397, 398]. The ligand will then bind to the receptor binding site and the radioactive signal from the bound ligand can then be detected by autoradiographic methods, in this study by using films. Together these methods, ISH, ICC and receptor autoradiography enable a good understanding of the origin, distribution and location of a specific receptors and hence, of their physiological functioning [397]. An I^{125}-orithine vasotocin analog (I^{125}-d$(CH_2)_5$[Tyr(Me)^2,Thr^4,Orn^8](^{125}I)Tyr^9-NH_2]; Perkin Elmer) or the linear I^{125} AVP-A tracer (I^{125}-d$(CH_2)_5$[Tyr(Me)]-AVP; Perkin Elmer) was used for the OXT and AVP receptor binding studies respectively.

2.8.2 Method

Slides with unfixed coronal brain sections of interest were removed from storage at -80^{o}C, left at 4^{o}C for 30 min and for a further 30 min at room temperature. They were then put into glass troughs and fixed for 2 min in 0.1% paraformaldehyde followed by rinses in 50mM tris buffer (Sigma; 2x10 min). Next, slides were incubated for 60 min in 50mM tris buffer with 10mM $MgCl_2$ (Sigma), 0.1% BSA (Sigma) and radioactive tracer (2000cpm/10µl; Perkin Elmer). Slides were rinsed with tris $MgCl_2$ buffer (4x5 min) followed by a 30 min spinning wash in tris $MgCl_2$ buffer. Finally they were dipped into distilled water before being dried with cold air (from a hairdryer). In a dark room with a red light on, the slides were arranged onto a cassette and film (Kodak BioMaxMR) laid on top. The cassette was then closed and stored in the dark room for the desired exposure time.

2.8.3 Quantification Technique

Receptor binding was quantified using the NIH Image program (Image J 1.31; National Institute of Health) as grey density minus background for specific brain areas of interest (see appendix for definition of these areas). Deduction of background means the measurement reflects specific binding only. Means were calculated for each individual rat using brain slices with areas of interest comparable to each other (4 sections per rat).

2.9 Progesterone Radioimmunoassay (RIA)

2.9.1 General principles

This assay enables accurate measurement of serum progesterone concentrations by using a radioactively labelled antigen (I^{125} labelled progesterone in this case) which will compete with a non-radioactively antigen for a specific number of antibody binding locations. Once free and bound antigens have been separated by decanting, the amount of radioactively bound antigen can then be measured using a gamma counter and is inversely proportional to the concentration of non-radioactive antigen present in the sample. The assay sensitivity is 0.12ng/ml.

2.9.2 Method

Prior to performing the assay, standards and control solutions were reconstituted by adding 0.5ml of deionised water to each, except standard A which required 1 ml. Standards and controls were included in each assay run. The values from the standards were used to form a standard curve from which the concentration of progesterone in unknown samples was read (an example of a standard curve is in

figure 3). Controls were included in order to check the validity of the standard curve formed.

Two plain tubes were labelled for total counts and the anti-progesterone-coated tubes were labelled in duplicate for standards, controls and samples. To each appropriately labelled tube, 25µl of standard, control or sample was pipetted to the bottom. Immediately, 500 µl of progesterone (I^{125}) reagent was pipetted into each tube and the rack gently shaken before incubating all tubes at 37 ^{o}C for 70 min. All tubes were decanted, except total counts, and after being blotted on absorbent paper, left inverted to drain completely for 2 min. Once drained, all tubes were counted using a gamma counter for 1 min.

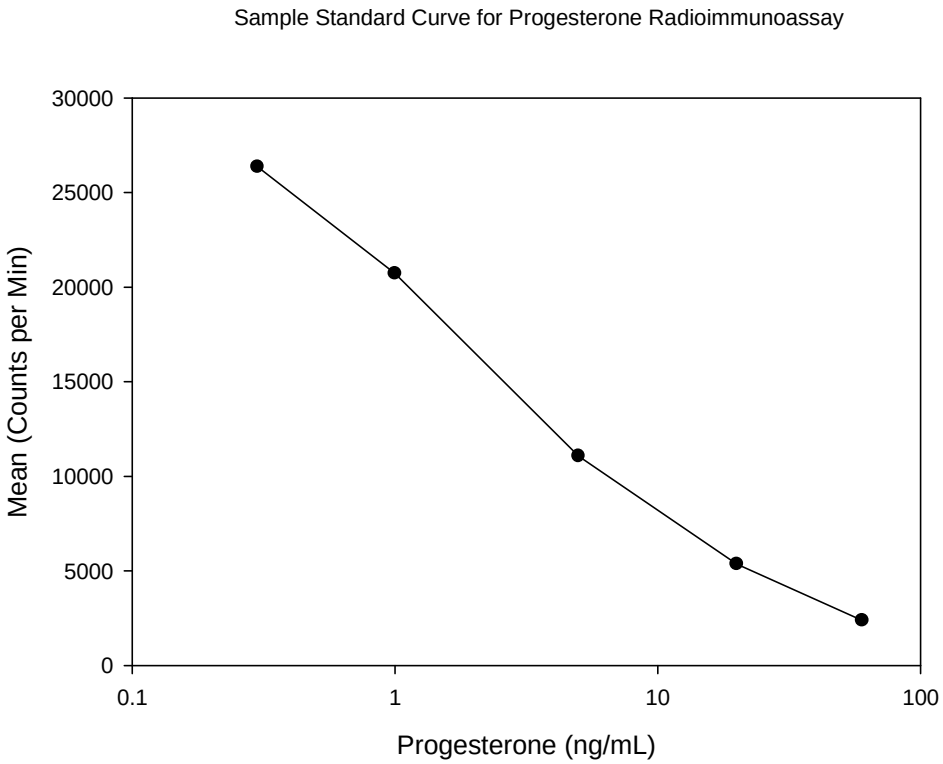


Figure 2.3: Example of a standard curve for progesterone radioimmunoassay. Values used are from the data given by Diagnostics Systems Laboratories as typical Active Progesterone Standard Curve Data (DSL 3900).

Chapter Three: Maternal aggression and pup removal

3.1 Introduction

Maternal aggression is an important maternal behaviour generally defined as the protection of the offspring from a harmful intruder [169, 175, 176, 180, 399]. To this end there must be some balance in expressing maternal aggression between protecting the offspring and harm that may come to the mother during the aggressive encounter [54].

In lactating mice, maternal aggression is significantly decreased if pups are removed for at least 5h but maternal aggression can be re-instated by the re-introduction of pups for as little as 5 min [6, 169]. Similar findings have been reported for lactating rats where pup removal for 4h significantly decreased attack and bite number and significantly increased attack latency [179, 190]. By 24h after pup removal maternal aggression was entirely eliminated in rats [179, 190]. As in mice, it was observed that maternal aggression could be reinstated if lactating rats were re-exposed to pups although the time period required was substantially longer (at least 1h) [179]. Studies in hamsters however, have shown that 6h of pup removal had little or no effect on maternal aggression [175]. It was suggested that this was due to the fact that lactating hamsters were tested in their home cage and therefore the olfactory cues remaining from pup odour on the bedding meant maternal aggression did not diminish [175]. However, this seems unlikely as both mice and rats in the studies described above were also tested in their home cages [6, 169]. Until further experiments are performed a better hypothesis to test would be that for maternal aggression in the hamster, pup cues do not play such an important role in reinforcing maternal aggression as they do in rats and mice [175]. In support of this is the

observation that maternal aggression in lactating hamsters does not diminish as the lactation period progresses in contrast to rats and mice, for which research has shown tactile and olfactory cues from pups are essential in maternal aggression regulation [188].

Nulliparous mice that have undergone thelectomy (surgical removal of nipples) prior to mating or parturition (i.e. pre-partum) exhibited significantly reduced maternal aggression compared to mice given post-partum thelectomy after 48h of suckling experience [195]. This indicates that suckling experience (of at least 48h) is essential in mice for the initiation but not maintenance of maternal aggression [195]. It is olfactory, visual and auditory cues from the pups which are important for maternal aggression maintenance as lactating mice whose pups were present behind a mesh wire (i.e. no physical contact between mother and pups) still exhibited normal maternal aggression levels towards a novel conspecific intruder [6]. Olfactory cues also appear to help with identification of the intruder. Studies have shown no maternal aggression is displayed to a male if the resident lactating mouse has previously been exposed to this individual behind a wire mesh (again preventing physical contact) for at least 7 days (lactation days 2-7) [6]. It may be advantageous not to attack a familiar male as in the wild it has been observed that mice communally rear offspring [6].

In the rat, suckling does not appear to be as important for the initiation of maternal aggression. Pre-partum thelectomy does not affect maternal aggression at late pregnancy or postpartum, however suckling must provide some important sensory stimulation necessary for maternal aggression as complete anaesthesia of the nipple and surrounding skin severely reduced or abolished maternal aggression in the

Long Evans strain of rat [183, 190]. Olfactory cues for rats, as in mice, are important in the regulation of maternal aggression. Complete removal of the olfactory bulbs (BOBX) results in abolishment of maternal aggression but this may be a consequence of poor maternal behaviour rather than an inability to detect olfactory cues [225]. However BOBX does not allow distinction between volatile and non volatile (pheromonal) olfactory cues, if lactating rats are subjected to $ZnSO_4$ application which eliminates only volatile cues by destroying the olfactory epithelium. In this case little or no effect is observed on maternal aggression unless tested later in lactation where it significantly reduces maternal aggression [181, 188, 225]. It has been proposed that maternal aggression during early lactation is regulated by both hormones of the peri-partum period and sensory cues from pups. As the lactation period progresses and hormonal levels normalise, the primary maternal aggression drive is from pup chemosensory cues [181, 184]. Nevertheless the reduction in maternal aggression observed in both rats and mice following pup removal does highlight the point that maternal aggression is not entirely regulated by the hormonal changes of the peri-partum period; it must also rely on reinforcement from pup cues, both somato- and chemosensory.

It has been established that it is possible to induce maternal behaviour and maternal aggression in virgin female rats by exposing them to pups for 24h a day for at least 7 days without any hormonal manipulations [10]. However although the level of maternal behaviour is similar to lactating rats, the level of maternal aggression is never as high [3, 173, 400]. In contrast, virgin female mice are observed to express maternal behaviour towards 1 day old pups spontaneously [182]. Further to this, these pup-sensitized mice also display maternal aggression towards a novel conspecific

[182]. If pups are removed from these pup-sensitized virgin female mice, maternal aggression diminishes in the same manner as in lactating mice although within only 1h compared to the 5h separation required by lactating mice [182]. As in mice, pup-sensitized ovariectomised rats show maternal aggression towards a conspecific male [174]. These studies provide evidence that the expression of maternal behaviour, and hence learning to interact with pups and interpret pup signals, is important in the development of maternal aggression as well as hormonal influences.

Mapping of both Fos and pCREB expression has been reported with increases in many specific brain areas following the display of maternal aggression in both mice and rats [12, 67, 188, 194]. In lactating mice, the number of Fos positive cells was observed to significantly increase in the claustrum, BnST, MPOA, PVN, MeA and cortical amygdala following maternal aggression [12]. pCREB expression in lactating mice which displayed aggression during a maternal aggression test was reported to significantly increase only in the ventrolateral PAG and LS compared to non aggressive lactating mice [12]. These results are in concordance with Hansen and Gammie (2005, [194]) who observed significant increases in Fos expression in the BnST (dorsal and ventral), PVN, amygdaloid complex (central, basolateral and medial), MPOA, cingulate cortex and OBs (mitral and granular call layers) in aggression tested lactating mice compared to virgin females. Furthermore, examination of early growth response factor expression (Egr-1) showed significantly increased activation in the PVN, lateral PAG, MeA, CeA, BlA, anterior, lateral and ventromedial hypothalamus in aggression tested lactating mice compared to untested mice [196]. Aggression tested lactating rats similarly showed increases in Fos expression in the BnST, PVN, MeA and PAG regions [188]. Together these results

provide evidence for the brain regions important in the regulation of maternal aggression and highlight the neural circuitry of maternal aggression.

Lactating rats not only display fierce aggressive behaviour but have also been extensively reported to exhibit reduced anxiety on the EPM, in conflict tests and open field paradigms [29, 31-33]. They also express a reduced fear response to a sudden noise [42]. However, little is known of the effect of maternal aggression on the display of anxiety, as most studies have examined both separately but never in relation to one another.

In this chapter, the first aim was to clearly define the changes to specific maternal aggression behavioural components following pup removal for increasing duration in lactating rats. The second aim was to discover whether the level of activation in specific brain regions correlates with the expression of specific maternal aggression behavioural components. This was done by examining Fos expression in specific brain regions linked to maternal aggression in the rats from the pup removal experiment and testing if the number of Fos positive cells correlates with the duration of a specific maternal aggression behavioural component. Pup-sensitized virgin female rats were also tested in these paradigms to allow us to compare the effects of hormone and pup stimulation versus pup stimulation only on maternal aggression expression and brain activation. This experiment was done with the assistance of Honours student, Kathryn Cruickshank. The final aim was to examine whether the lactating rat being in the aggressive state results in a lower anxiety behaviour profile. This was investigated by observing the behaviour of lactating rats on the EPM following the exhibition of aggressive behaviour.

3.2 The effect of pup removal on the expression of maternal aggression in lactating and pup-sensitised virgin female rats

3.2.1 Method

For lactating rats (Dam), their entire litter of pups was left (n=8) or removed from their home cage 2 (n=7), 6 (n=7) or 24h (n=8) prior to a 30 min maternal aggression test. Virgin female rats (Virgin) underwent the pup-sensitization process (described in chapter 2). On the day after virgins were defined as 'fully maternal', they were randomly assigned to one of two groups. The pup-sensitization observation was performed for both groups as before and one group was then immediately exposed to a 30 min maternal aggression test (n=7). The other group had all donor pups removed immediately after the pup-sensitization test and 2h later was subjected to a 30 min maternal aggression test (n=9). Ninety min following the start of the maternal aggression test, rats were anthaesthetised and perfused with 4% paraformaldehyde. The brains from perfused rats were collected and processed for Fos ICC.

The number of Fos positive cells were counted in the LS, BnST, MPOA, SON, MeA, CeA, PVN and PAG according to the ICC quantification technique described in chapter 2.

3.2.1.1 Statistics

A one way ANOVA was performed to compare the effects of treatment (0, 2, 6 or 24h pup removal) on the all groups for behaviour and Fos expression in the LS, BnST, MPOA, SON, MeA, CeA, PVN and PAG with a Holm-Sidak post hoc test to determine which groups were statistically significantly different. If data were not normally distributed a one way ANOVA on ranks was performed using a Dunn's test post hoc instead. When $p \leq 0.05$, data were defined as significantly different.

3.2.2 Results

3.2.2.1 Pup-sensitization

A total of 26 virgin female rats began the pup-sensitization procedure. One rat immediately killed the pups in the first day of testing and was subsequently removed from the whole experiment. Of the remaining 25 virgin rats, 9 did not express any maternal behaviour after 13 days of testing and were removed from the study. The other 16 became maternal within an average of 7.37±0.7days so 61.5% of virgin female rats that started the pup-sensitization process were successfully pup-sensitized. There was no significant difference in latency to become maternal between the two groups chosen for the pup removal experiment ($p=0.15$, $t_{1.53}$). Another virgin female rat that became maternal following the pup-sensitization procedure was removed from the study because during maternal aggression testing this rat and the intruder killed the pups.

3.2.2.2 Aggressive behaviour

Attack behaviour expression in lactating rats changed dramatically following removal of pups (Fig. 3.1). The latency to attack was significantly decreased 6h (dam 6h; $p<0.001$, $H_5=31.99$) and 24h (dam 24h; $p<0.001$, $H_5=31.99$) following pup removal compared to dams with pups present (dam PP) but not by 2h (dam 2h; $p>0.05$, $H_5=31.99$; Fig. 3.2). Time spent displaying attack behaviour and attack number were also significantly lower in the dam 6h (attack duration - $p<0.001$, $H_5=30.67$; attack number – $p<0.001$, $H_5=29.65$) and dam 24h (attack duration - $p<0.001$, $H_5=30.67$; attack number – $p<0.001$, $H_5=29.65$) groups compared to dam PP but not dam 2h group (Fig. 3.2 and 3.3).

Pup sensitized virgin rats displayed significantly fewer attacks ($p<0.001$, $H_5=29.65$) and longer attack latencies ($p<0.001$, $H_5=31.99$) whether pups remained present (virgin PP) or had been removed for 2 hours (virgin 2h) compared to the dam PP group but no other dam group. Both groups also spent a significantly shorter time attacking the intruder compared to the dam PP group ($p<0.001$, $H_5=30.67$).

For other aggressive behaviours, the duration of biting behaviour was significantly reduced in the dam 2h ($p<0.001$, $H_5=22.11$; Fig. 3.3), dam 6h ($p<0.001$, $H_5=22.11$; Fig. 3.3) and virgin 2h ($p<0.001$, $H_5=22.11$; Fig. 3.3) groups but not the dam 24h ($p>0.05$, $H_5=22.11$; Fig. 3.3) or virgin PP ($p<0.001$, $H_5=22.11$; Fig. 3.3) groups compared to the dam PP group. Furthermore, although rearing duration was significantly lower in the dam 6h and virgin PP groups (vs dam PP; $p=0.002$, $H_5=19.32$), no change was observed in the dam 24h or virgin 2h groups vs the dam PP group ($p>0.05$, $H_5=19.32$; Fig. 3.3). Expression of pinning down ($p=0.003$, $H_5=17.86$) behaviour was only significantly lower by 24h following pup removal in the lactating rat compared to the dam PP group; but in the pup sensitized virgin group both the virgins with pup present and virgin with pups removed for 2h displayed significantly lower pinning down behaviour compared to the dam PP group ($p=0.003$, $H_5=17.86$). No significant difference was observed in clawing behaviour across all groups ($p=0.052$, $H_5=10.97$; Fig. 3.3). Duration in lunging behaviour was significantly different between all groups ($p=0.010$, $H_5=15.11$) but post hoc analysis was unable to determine between which specific groups.

3.2.2.3 Sniffing behaviour

Duration of sniffing was observed to be significantly different between all groups ($p<0.001$, $F_{(5,42)}=5.85$; Fig. 3.2). Two hours following pup removal, time spent

sniffing was significantly less than the dam PP group ($p=0.005$, $F_{(5,42)}=5.85$). This remained so for the dam 6h ($p=0.004$, $F_{(5,42)}=5.85$) and dam 24h ($p=0.004$, $F_{(5,42)}=5.85$) groups compared to the dam PP group. Pup-sensitized virgin spent significantly less time sniffing whether pups were present ($p=0.003$, $F_{(5,42)}=5.85$) or 2h after removal ($p=0.004$, $F_{(5,42)}=5.85$) compared to the dam PP group.

3.2.2.4 General behaviour

There was a significant difference in the duration of general behaviours between the all groups ($p<0.001$, $F_{(5,40)}=13.88$) with the dam PP group spending significantly less time displaying general behaviour than the dam 2h, 6h and 24h and virgin 2h groups (Fig. 3.4). The virgin PP group also displayed significantly less general behaviour than the dam 2h, 6h, and 24h groups but not the virgin 2h group (Fig. 3.4). Expression of exploring behaviour was higher in dam 24h and virgin 2h group compared to the dam PP group only ($p=0.002$, $H_5=19.43$). Duration of grooming self behaviour was significantly higher in the dam PP group compared to the dam 2h and 24h groups only (($p=0.048$, $F_{(5,41)}=2.47$; Fig. 3.4).

3.2.2.5 Maternal behaviour

The virgin PP group exhibited significantly more nesting behaviour than dam PP group ($p=0.006$, $T_{(7,8)}=79$; Fig. 3.4).

3.2.2.6 Response to aggression behaviour by the resident

There was a significant difference between all groups in duration of response to aggression behaviours ($p=0.044$, $H_5=11.40$; Fig. 3.4) but a Dunn's post hoc test was unable to determine between which specific groups.

3.2.2.7 Fos expression

Fos expression was observed to change significantly following different duration after pup removal in lactating rats. By 2h following pup removal, Fos expression was significantly lower in the BnST ($p<0.001$, $F_{(5,38)}=28.01$), PVN ($p=0.029$, $F_{(5,38)}=2.82$), MeA ($p=0.001$, $H_5=19.93$) and SON ($p<0.001$, $F_{(5,32)}=8.87$) compared to the dam PP group and remained so in the dam 6h and 24h groups (Fig. 3.5). However, although Fos expression in the PAG ($p=0.015$, $H_5=14.01$) was significantly lower than the dam PP group in the dam 6h, no significant difference was observed in the dam 24h group (Fig. 3.5). After 24h of pup removal, Fos expression within the LS ($p<0.001$, $H_5=26.60$), and CeA ($p<0.001$, $H_5=22.53$) was significantly lower than in the dam PP group (Fig. 3.5).

Fos expression in the BnST ($p<0.001$, $F_{(5,38)}=28.01$), LS ($p<0.001$, $H_5=26.60$), SON ($p<0.001$, $F_{(5,32)}=8.87$) and CeA ($p<0.001$, $H_5=22.53$) was significantly lower in both the pup sensitized virgin rat group with pups present and after 2h of pup removal compared to the dam PP group. Interestingly, only the pup sensitized virgin rats whose pups had been removed for 2h had significantly lower Fos expression in the PVN ($p=0.029$, $F_{(5,38)}=2.82$) and MeA ($p=0.001$, $H_5=19.93$) but only when compared to the dam PP group and not to the virgin PP group.

Significant difference for Fos expression was observed within the MPOA across all the dam groups ($p=0.034$, $H_5=12.08$; Fig. 3.5), but post hoc analysis was unable to determine between which specific groups.

3.2.2.8 Correlations of Fos expression with aggressive behaviour

Significant correlations between Fos expression in specific brain regions and duration of different components of aggressive behaviour were observed. Table 3.1

depicts the p values and r coefficients for each brain region against each specific behaviour (see appendix 6 for graphs). The BnST is one area highlighted as important in the aggression circuitry as almost all correlations were significant (positively with attack number, attack/biting/clawing/lunging/pinning down duration and negatively with attack latency; Table 3.1, Figs. 3.6-3.13); only clawing duration was not significant. Similarly, Fos expression within the LS, MeA and PAG were significantly correlated with most aggressive behaviours (positively with attack number, attack/biting/clawing/lunging/pinning down duration and negatively with attack latency; Table 3.1; see appendix 6) but not rearing or lunging for the LS, and not clawing or lunging for the MeA and PAG. The SON, PVN and CeA are also implicated as significant positive correlations were observed between Fos expression and lunging, Fos expression and attack/biting/pinning down duration or Fos expression and attack number/attack/pinning down respectively (Table 3.1;s. appendix 6). Fos expression in the SON, PVN and CeA was also negatively correlated with attack latency (Table 3.1; appendix 6). In the MPOA, Fos expression was not significantly correlated with any specific aggressive behaviour (Table 3.1; appendix 6).

Sniffing duration was also examined and significant positive correlations were observed with Fos expression within the BnST, LS, MeA, CeA and PAG (Table 3.1; appendix 6).

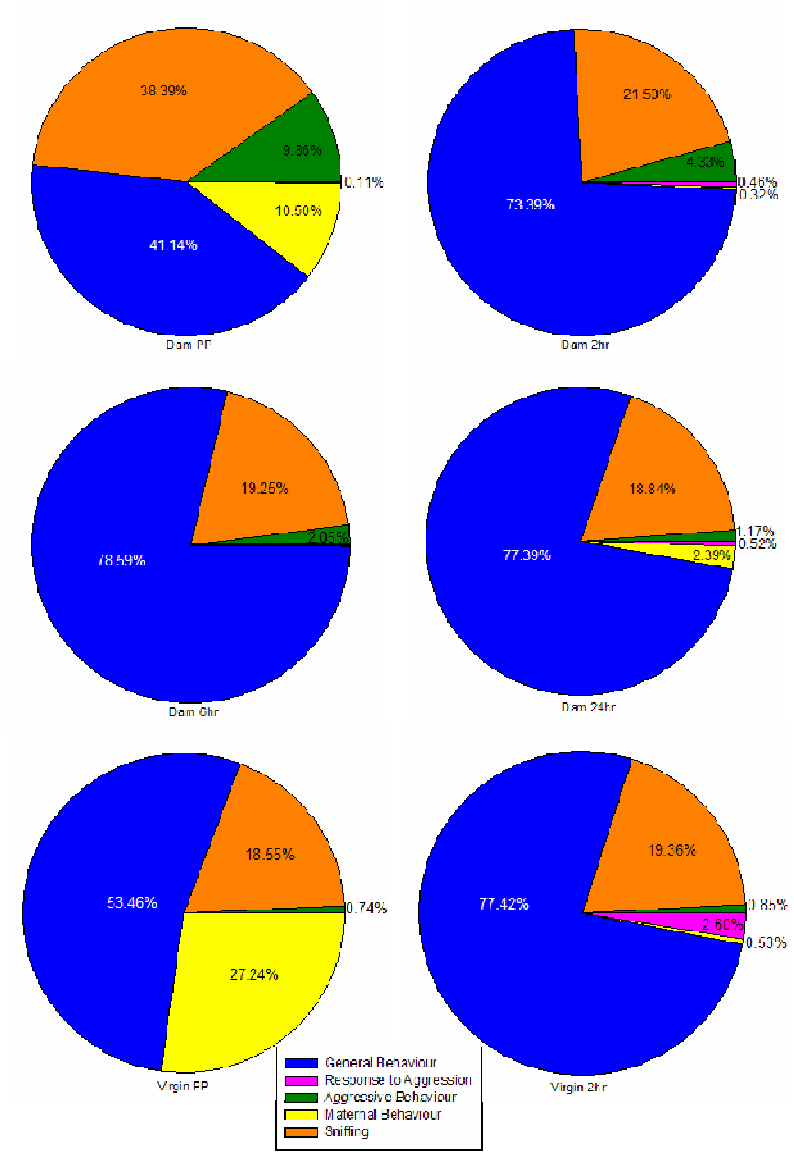


Figure 3.1: Mean percentage of total time spent exhibiting different behaviours by the resident rat during a maternal aggression test following pup removal. Virgin female rats were sensitized to donor pups until confirmed as expressing 'full maternal behaviour'. Pup-sensitized virgin and lactating rats were then subjected to a 30 min maternal aggression test with their pups present (Virgin PP, n=7; Dam PP, n=8) or 2h (Virgin 2h, n=9; Dam 2h, n=7), 6h (Dam 6h, n=7) or 24h (Dam 24h, n=8) after pups were removed. The average percentage of the total time of the 30 min maternal aggression test spent by the resident rat exhibiting aggressive (including attacks, bites, lunging), maternal (including pup moving and nursing), response to aggression (including freezing) and general (including exploring, eating and drinking) behaviours. In the groups where pups removed, maternal behaviour was defined as the digging in the nest or searching the cage for pups. As expected one can see in the dam PP group spent the greatest percentage of time exhibiting aggressive behaviour and sniffing behaviour which reflects investigation of the intruder. As duration of pup removal increases the percentage of time spent expressing aggressive behaviour lowers in the dam groups. Note that the percentage of time expressing aggressive behaviours in the dam 6h is comparable to the virgin PP and 2h groups indicating maternal aggression is diminishing following removal of pups. One other important feature to note is the higher percentage of maternal behaviour expression in the virgin PP group compared to the dam PP group confirming maternal behaviour is being expressed but pup-sensitization alone has not resulted in maternal aggression.

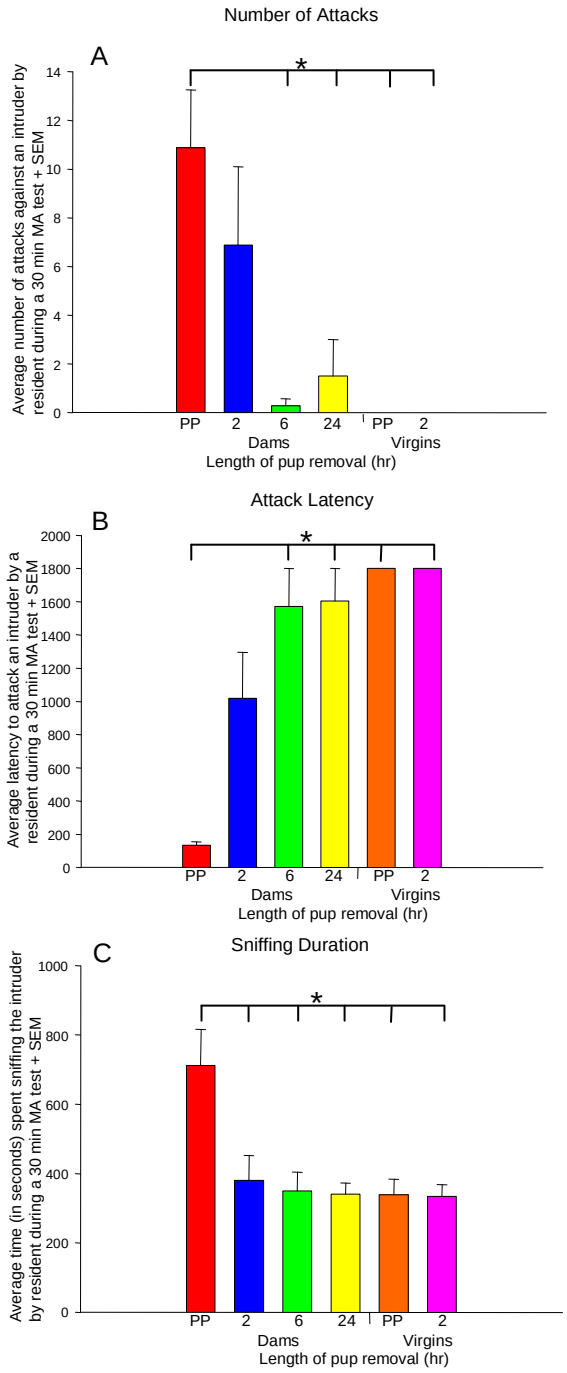


Figure 3.2: Average number of attacks, attack latency and duration of sniffing by the resident rat during a maternal aggression test following pup removal. Virgin female rats were sensitized to donor pups until confirmed as expressing 'full maternal behaviour'. Pup-sensitized virgin and lactating rats were then subjected to a 30 min maternal aggression test with their pups present (Virgin PP, n=7; Dam PP, n=8) or 2h (Virgin 2h, n=9; Dam 2h, n=7), 6h (Dam 6h, n=7) or 24h (Dam 24h, n=8) after pups were removed. The average number of attacks (A) and latency to attack (B) exhibited by the resident rat towards a novel intruder are depicted. The average duration (secs) the resident rat spent sniffing the intruder rat during the 30 min maternal aggression test is shown in C. A one-way ANOVA was used to compare groups followed by a Holm-Sidak post hoc test. If data were not normally distributed, a one-way ANOVA on ranks was performed followed by a Dunn's post hoc test. Data are represented as mean + SEM. *p≤0.05

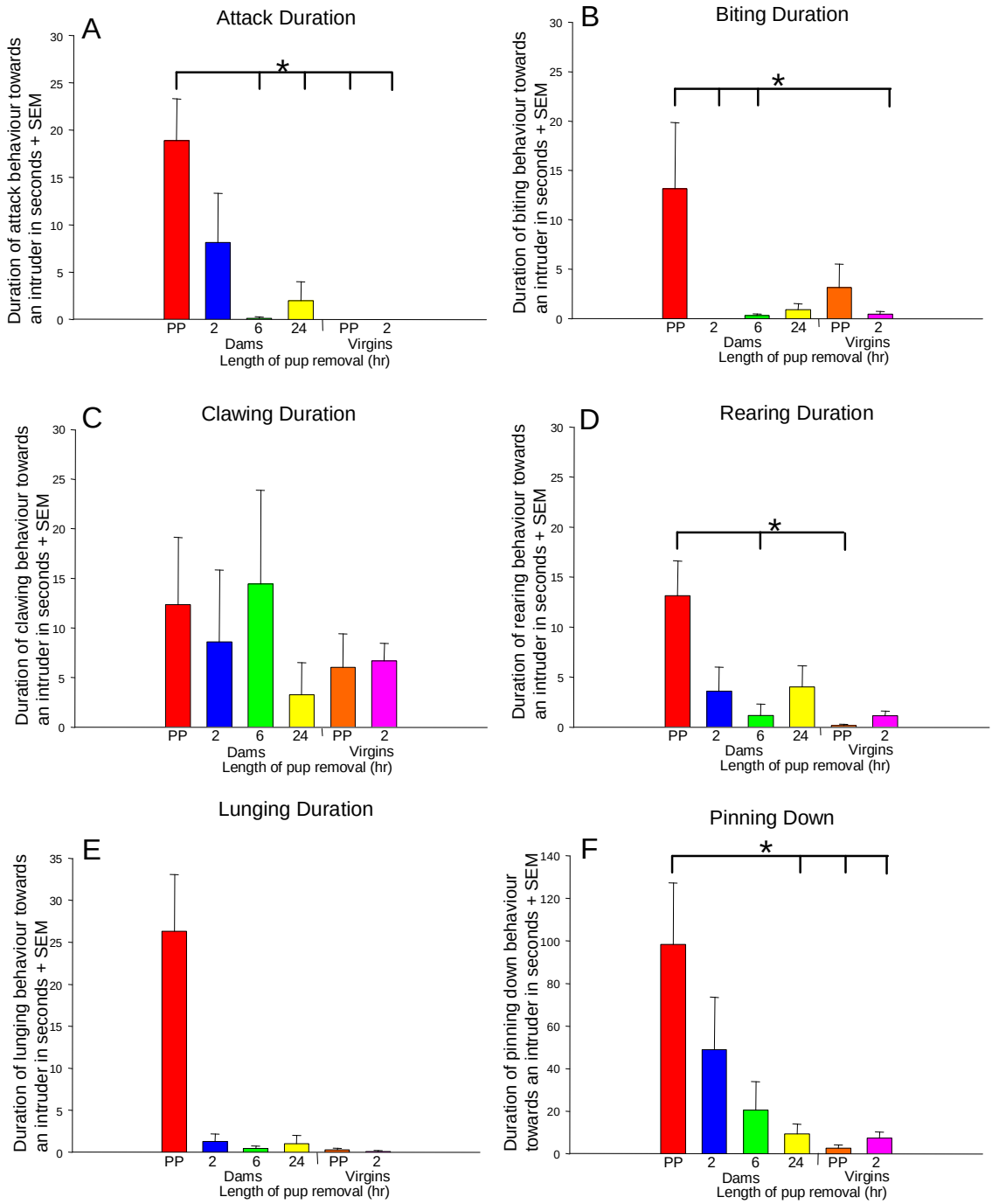


Figure 3.3: Average duration of specific aggressive behaviours expressed by the resident rat during a maternal aggression test following pup removal. Virgin female rats were sensitized to donor pups until defined as expressing 'full maternal behaviour'. Pup-sensitized virgin and lactating rats were then subjected to a 30 min maternal aggression test with their pups present (Virgin PP, n=7; Dam PP, n=8) or 2h (Virgin 2h, n=9; Dam 2h, n=7), 6h (Dam 6h, n=7) or 24h (Dam 24h, n=8) after pups were removed. The average duration (secs) of attack (A), biting (B), clawing (C), rearing (D), lunging (E) and pinning down (F) behaviour exhibited by the resident rat towards a novel intruder are depicted. A one-way ANOVA was used to compare groups followed by a Holm-Sidak post hoc test. If data were not normally distributed, a one-way ANOVA on ranks was performed followed by a Dunn's post hoc test. Data are represented as mean + SEM. *p≤0.05

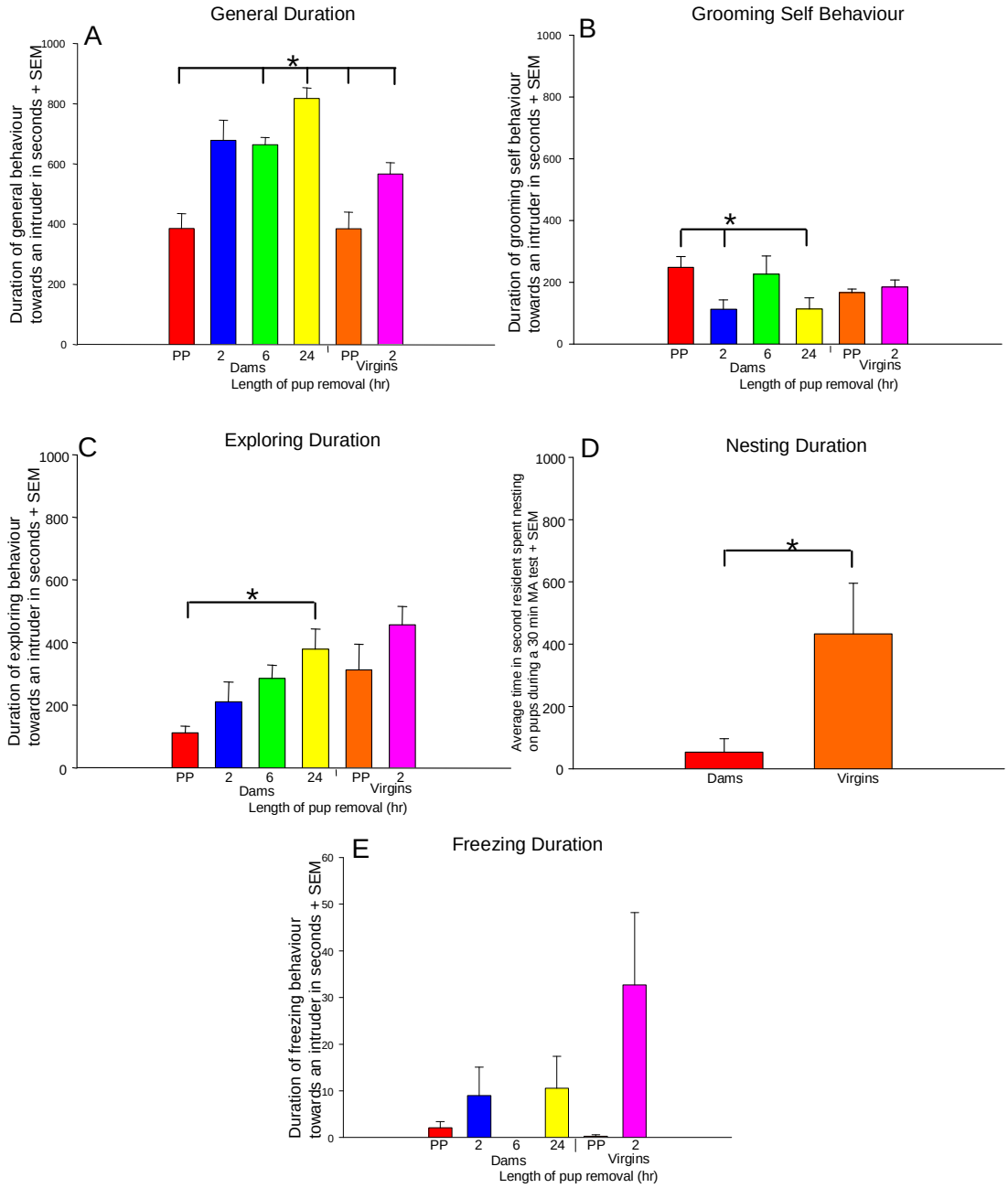


Figure 3.4: Average duration of specific general, maternal or response to aggression behaviours expressed by the resident rat during a maternal aggression test following pup removal. Virgin female rats were sensitized to donor pups until defined as expressing 'full maternal behaviour'. Pup-sensitized virgin and lactating rats were then subjected to a 30 min maternal aggression test with their pups present (Virgin PP, n=7; Dam PP, n=8) or 2h (Virgin 2h, n=9; Dam 2h, n=7), 6h (Dam 6h, n=7) or 24h (Dam 24h, n=8) after pups were removed. The average duration (secs) of general (A), grooming self (B), exploring (C), nesting (D) and freezing (E) behaviour exhibited by the resident rat during a 30 min maternal aggression test. A one-way ANOVA was used to compare groups followed by a Holm-Sidak post hoc test. If data were not normally distributed, a one-way ANOVA on ranks was performed followed by a Dunn's post hoc test. Data are represented as mean + SEM. *p≤0.05

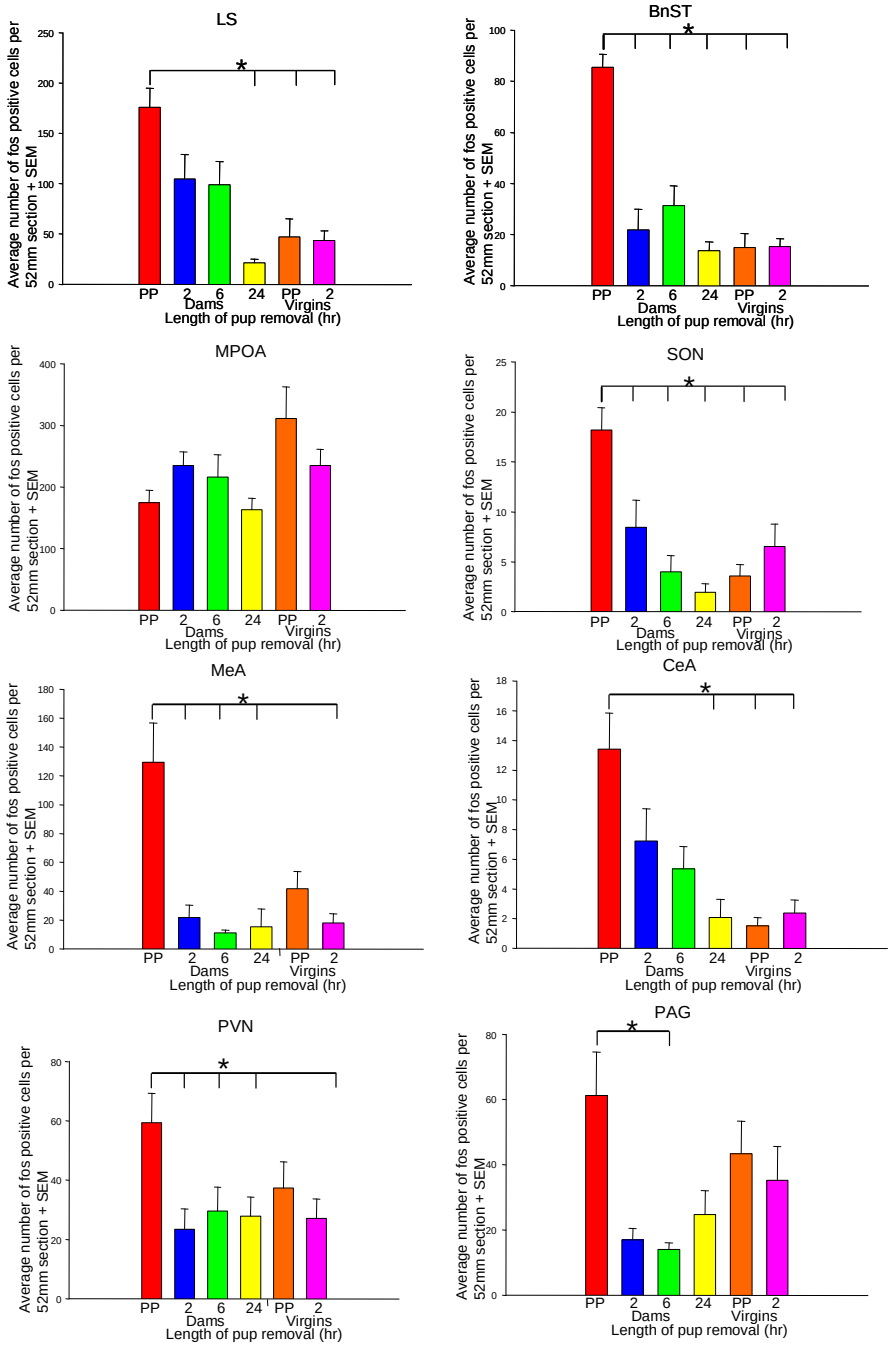


Figure 3.5: Average number of Fos positive cells in specific brain areas of the resident rat during a maternal aggression test following pup removal. Virgin female rats were sensitized to donor pups until defined as expressing 'full maternal behaviour'. Pup-sensitized virgin and lactating rats were then subjected to a 30 min maternal aggression test with their pups present (Virgin PP, n=7; Dam PP, n=8) or 2h (Virgin 2h, n=9; Dam 2h, n=7), 6h (Dam 6h, n=7) or 24h (Dam 24h, n=8) after pups were removed. The number of cells expressing Fos was counted in the lateral septum (LS), bed nucleus of stria terminalis (BnST), medial preoptic area (MPOA), supraoptic nucleus (SON), medial and central amygdala (MeA and CeA respectively), paraventricular nucleus (PVN) and periaqueductal grey (PAG) of the resident rat following a 30 min maternal aggression test after pup removal for increasing time lengths. A one-way ANOVA was used to compare groups followed by a Holm-Sidak post hoc test. If data were not normally distributed, a one-way ANOVA on ranks was performed followed by a Dunn's post hoc test. Data are represented as mean + SEM.*p≤0.05.

Brain Region / Behaviour	BnST	LS	MPOA	SON	MeA	CeA	PVN	PAG
Attack Number	***p=0.005 r^2=0.50***	***p=0.01 r^2=0.45***	p=0.43 r^2=0.16	p=0.10 r^2=0.44	***p=0.01 r^2=0.46***	***p=0.02 r^2=0.42***	p=0.06 r^2=0.35	***p=0.001 r^2=0.57***
Attack Latency	***p=0.001 r^2=-0.56***	***p=0.001 r^2=-0.55***	p=0.50 r^2=-0.04	***p=0.04 r^2=-0.41***	***p=0.002 r^2=-0.55***	***p=0.003 r^2=-0.53***	***p=0.01 r^2=-0.47***	***p=0.0003 r^2=-0.63***
Attack Duration	***p=0.003 r^2=0.52***	***p=0.01 r^2=0.46***	p=0.51 r^2=0.13	p=0.07 r^2=0.36	***p=0.01 r^2=0.46***	***p=0.02 r^2=0.43***	***p=0.04 r^2=0.39***	***p=0.0008 r^2=0.59***
Biting Duration	***p=0.01 r^2=0.45***	***p=0.02 r^2=0.40***	p=0.47 r^2=0.15	p=0.15 r^2=0.29	***p=0.01 r^2=0.45***	p=0.10 r^2=0.31	***p=0.03 r^2=0.41***	***p=0.0001 r^2=0.65***
Clawing Duration	p=0.08 r^2=0.33	***p=0.03 r^2=0.39***	p=0.51 r^2=0.13	p=0.43 r^2=0.16	p=0.08 r^2=0.34	p=0.37 r^2=0.17	p=0.43 r^2=0.16	p=0.05 r^2=0.37
Lunging Duration	***p=0.04 r^2=0.37***	p=0.14 r^2=0.27	p=0.44 r^2=0.16	***p=0.03 r^2=0.42***	p=0.10 r^2=0.31	p=0.21 r^2=0.24	p=0.11 r^2=0.31	p=0.06 r^2=0.35
Pinning Down Duration	***p=0.001 r^2=0.56***	***p=0.004 r^2=0.50***	p=0.74 r^2=0.07	p=0.09 r^2=0.34	***p=0.01 r^2=0.47***	***p=0.03 r^2=0.39***	***p=0.045 r^2=0.38***	***p=0.004 r^2=0.52***
Rearing Duration	p=0.11 r^2=0.30	p=0.23 r^2=0.23	p=0.93 r^2=0.02	p=0.21 r^2=0.26	***p=0.01 r^2=0.45***	p=0.25 r^2=0.22	p=0.26 r^2=0.22	***p=0.01 r^2=0.46***
Sniffing Duration	***p=0.009 r^2=0.47***	***p=0.001 r^2=0.56***	p=0.31 r^2=0.21	p=0.34 r^2=0.19	***p=0.004 r^2=0.53***	***p=0.0006 r^2=0.60***	p=0.08 r^2=0.34	***p=0.003 r^2=0.54***

Table 3.1: The p values and r coefficients for correlations between Fos expression within specific brain regions and specific behaviours of a lactating and pup-sensitized rat following a maternal aggression test. Virgin female rats were sensitized to donor pups until defined as expressing 'full maternal behaviour'. Pup-sensitized virgin (n=16) and lactating (n=30) rats were then subjected to a 30 min maternal aggression test with their pups present. The purpose of these correlations was to compare expression of a specific aggressive behaviour with Fos synthesis in specific brain regions linked with maternal aggression hence the data of pup-sensitized virgin and lactating rats was combined. The differences seen in behaviour and Fos expression between pup-sensitized virgin and lactating rats in Figs 3.1-3.5 therefore should be reflected in the correlations. It would be expected that less time spent expressing a specific aggressive behaviour would correlate with less Fos expression in specific brains regions. Abbreviations lateral septum (LS), bed nucleus of stria terminalis (BnST), medial preoptic area (MPOA), supraoptic nucleus (SON), medial and central amygdala (MeA and CeA respectively), paraventricular nucleus (PVN) and periaqueductal grey (PAG).

3.3 The effect of maternal aggression on the performance of a lactating rat in the elevated plus maze

3.3.1 Method

Between lactation days 3 to 7, SD rats were subjected to a 10 min maternal aggression test immediately followed by 5 min exposure to an EPM task (n=9) or a cage disturbance (as a control) 10 min prior to exposure to the EPM for 5 min (n=9). Lactating rats were anaesthetised and perfused with 4% paraformaldehyde 90 min after the start of maternal aggression test and the brain collected and processed for Fos ICC. Fos expression was counted and quantified in the LS, BnST, MPOA, SON, MeA, CeA and PVN brain regions using the technique described in chapter 2.

3.3.2 Results

3.3.2.1 Aggressive behaviour

The aggressive behaviour displayed by the aggression tested lactating rats was comparable to the level of aggression observed in lactation day (LD) 3-7 rats the experiment in which from maternal aggression was studied through pregnancy, parturition and lactation (chapter 4). The average number of attacks in 10 min was 2.11±0.8 (LD4-7 3.80±0.6; p=0.18, t_{12}=-1.43) and the average latency to attack was 252.14±61.7 secs (LD 3-7 146.1±42.2 secs; p=0.27, $t_{(5,7)}$=25.0).

3.3.2.2 Behaviour on the elevated plus maze

Aggression tested (AT) rats made significantly more entries into either open or closed arms overall (p=0.022, t_{16}=2.54; Fig. 3.6). They were significantly more likely to enter the open arms of the maze (p=0.031, t_{16}=2.37; AT=5.50±0.9 entries, non aggression-tested rats (NAT)=2.88±0.6 entries) although there was no significant difference in the number of entries into the closed arms (p=0.17, $t_{(8,10)}$=60.0; Fig.

3.6). The AT group also spent significantly more time in the open arms of the maze ($p=0.019$, $t_{(8,10)}=49.0$; AT=47.84±8.9sec, NAT=18.10±4.0sec) and significantly less time on the closed arms ($p=0.008$, $t_{16}=-3.03$; AT=230.68±10.7sec, NAT=271.58±7.0sec) than the NAT group (Fig. 3.6). Locomotion (cm/s) in the open arm was significantly increased in the AT group compared to the controls ($p=0.043$, $t_{16}=2.20$; AT=7.03±0.5cm/s, NAT=5.13±0.7cm/s) although there was no significant difference in the closed arms ($p=0.23$, $t_{16}=1.25$; Fig. 3.6). The total distance travelled (cm) was also significantly increased in the AT group overall ($p=0.012$, $t_{15}=2.86$) and in the open ($p=0.002$, $t_{(8,9)}=40.0$) but not in the closed arms ($p=0.399$, $t_{(8,9)}=66.0$) when compared to the NAT group (Fig. 3.6).

There were no significant differences between the groups in the latency to enter either open ($p=0.27$, $t_{16}=-1.15$) or closed arms ($p=0.40$, $t_{16}=0.87$; Fig. 3.6). No significant difference in time spent displaying rearing ($p=0.46$, $t_{16}=0.75$) behaviours between the two groups was observed, although there was a trend for the NAT group to spend more time grooming themselves ($p=0.054$, $t_{16}=-2.08$) than the AT rats in the closed arms (Fig. 3.7). Neither group displayed these behaviours in the open arms.

3.3.2.3 Fos expression

Fos expression was significantly higher in AT rats in the BnST ($p=0.023$, $t_{16}=2.51$), LS ($p=0.046$, $T_{(8,10)}=53.0$), MPOA ($p=0.007$, $t_{13}=3.18$) and MeA ($p=0.002$, $t_{(8,9)}=40.0$) compared to NAT rats (Fig. 3.8). There was no significant difference in Fos expression in the SON ($p=0.93$, $t_{(3,10)}=22.0$), CeA ($p=0.13$, $T_{(8,10)}=58.5$) or PVN ($p=0.098$, $t_{12}=1.79$) between the two groups (Fig. 3.8).

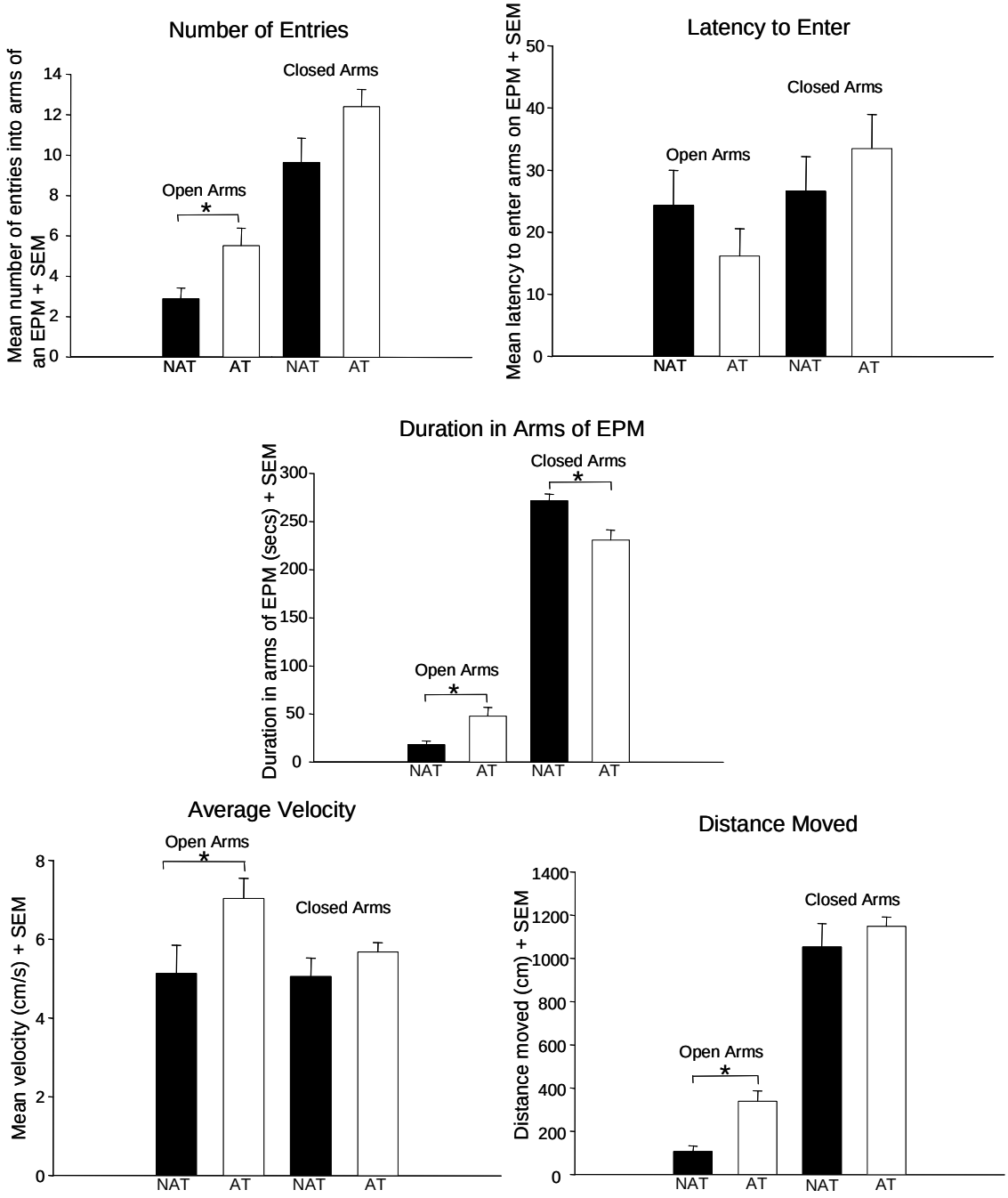


Figure 3.6: Number of entries, latency to enter, duration and mean velocity on an elevated plus maze by a lactating rat following a maternal aggression test. The average number of entries into the open and closed arms by a lactating rat during a 5 min test on an elevated plus maze (EPM). The aggression tested group (AT; n=9) received a 10 min maternal aggression test with a novel intruder in their home cage with their pups present prior to the EPM test, the non-aggression tested group (NAT; n=9) had a cage disturbance and were then left undisturbed 10 min prior to the test on the EPM. The mean duration (secs), average velocity (cm/s) and distance moved (cm) in the open and closed arms for lactating rats on an EPM was also examined. Data are represented as mean + SEM. *p≤0.05

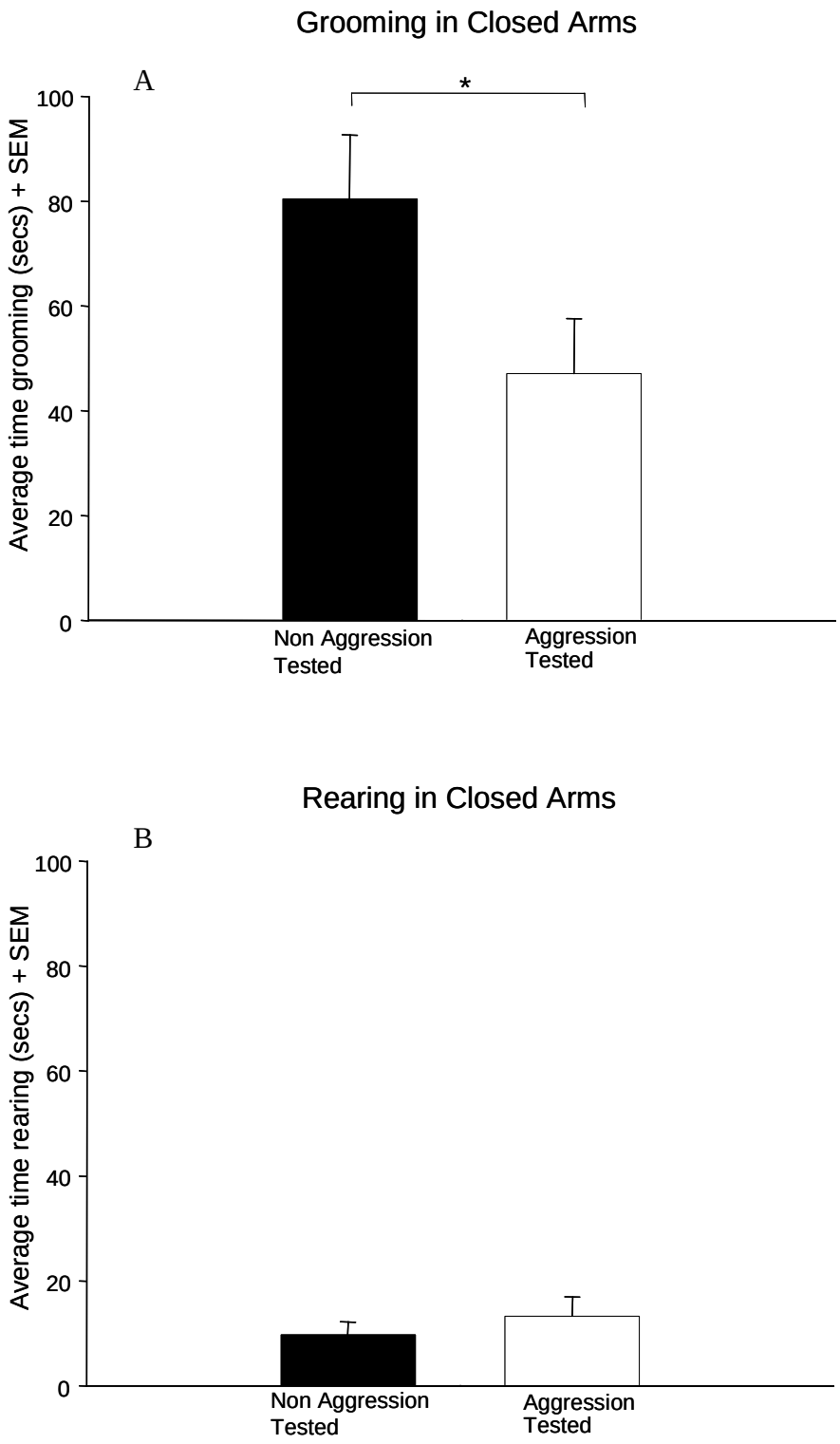


Figure 3.7: Average time spent by a lactating rat spent exhibiting different behaviours on an elevated plus maze following a maternal aggression test. The mean time (secs) for a lactating rat spent grooming (A) or rearing (B) in the closed arms on an elevated plus maze during a 5 min test. The aggression tested group (n=9) received a 10 min maternal aggression test with a novel intruder in their home cage with their pups present prior to the EPM test, the non-aggression tested group (n=9) had a cage disturbance and were then left undisturbed 10 min prior to the test on the EPM. Data are represented as mean + SEM. *p≤0.05

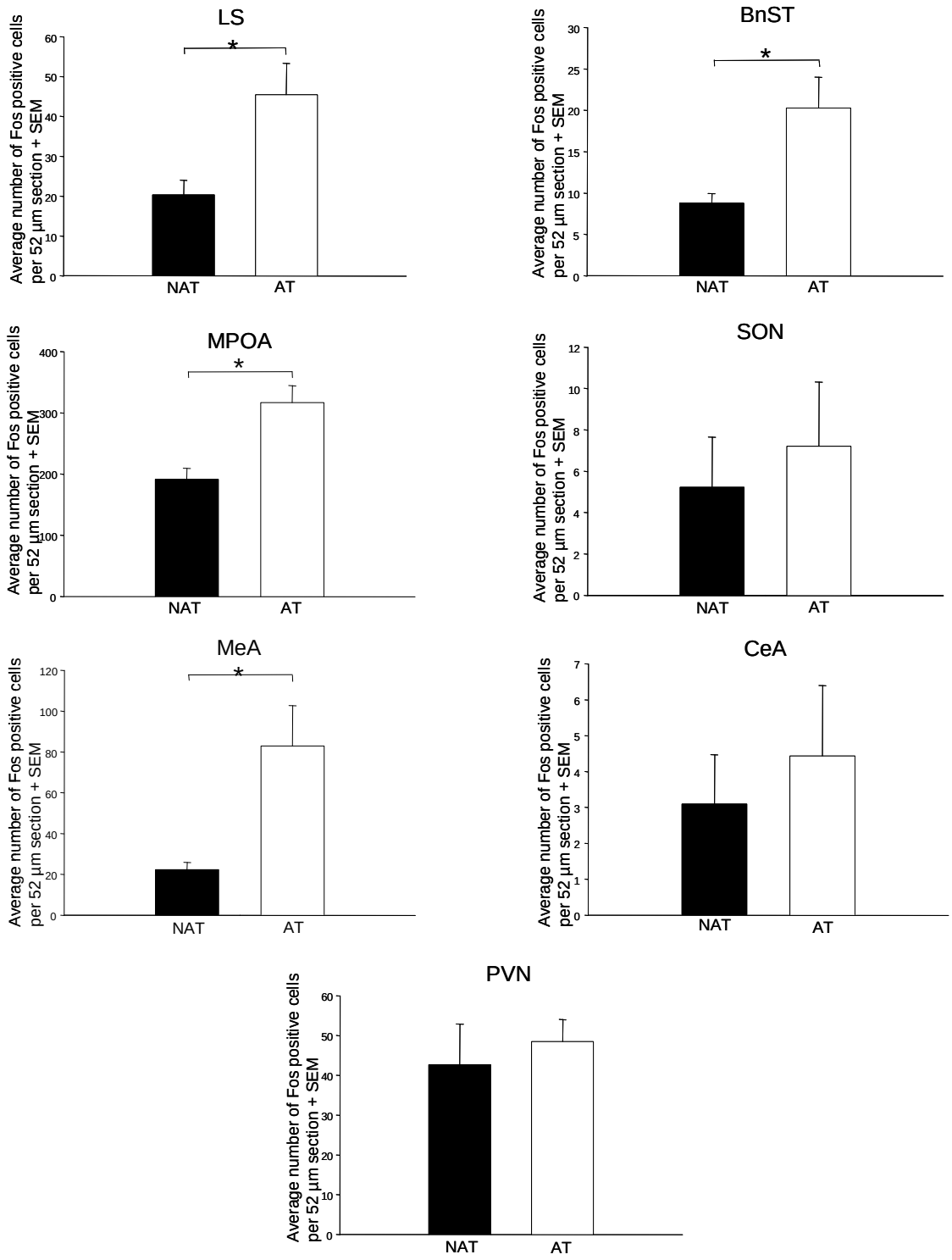


Figure 3.8: Average number of Fos positive cells in specific brain areas of a lactating rat following a maternal aggression and elevated plus maze test. Fos positive cells were quantified in the lateral septum (LS), bed nucleus of stria terminalis (BnST), medial preoptic area (MPOA), medial and central amygdala (MeA and CeA respectively), paraventricular nucleus (PVN) and supraoptic nucleus (SON) in a lactating rat following a maternal aggression and elevated plus maze (AT; n=9) test compared to a lactating rat that only performed the EPM test (NAT; n-9). Data are presented as mean + SEM. *p≤0.05

3.4 Discussion

In this study, it is observed that maternal aggression gradually diminishes in dams with lengthening separation from pups. From our study, pup removal for at least 6h is required for the most aggressive behaviour, attack, to be significantly reduced. Attack number, latency and duration were exhibited at levels similar to dams with pups present in dams 2h after their pups were removed. By 6h after pup removal attack number, latency and duration were all significantly lower suggesting there is a switch occurring in the control of the maternal aggression circuitry. Other studies have observed a significant decrease in maternal aggression 4h after pup removal in Long Evans female rats which would further narrow the time window in which this switch occurs [179]. Thus results from this and previous studies demonstrate that between 4h and 6h after pup removal, the attack component of maternal aggression is switched off. It is important to note that it was not until 24h following pup removal that most components of aggressive behaviour were expressed for significantly less time than by dams with pups present, similar to levels observed in lactation day 21 rats (chapter 4) and previous studies [190]. These behavioural results reveal that maternal aggression changes dramatically after the removal of pups and that the somatosensory and/or chemosensory cues from pups are essential in the maintenance of maternal aggression in lactating rats.

Examination of Fos expression in the brain and its correlation to aggressive behaviour highlighted the main brain regions involved in the maternal aggression control circuitry. The BnST was one region implicated as important in the maternal aggression circuitry; Fos expression was significantly lower in the maternal aggression test 2h after pup removal along with significant correlations between the

level of Fos expression within the BnST and duration of most aggressive behaviours (except for clawing). Significant correlations were also observed with attack latency and number. Fos expression within the BnST has already been observed to be significantly higher following maternal aggression testing in both mice and rats but this is the first study to show the level of activation is positively correlated with more aggressive behaviour display [12, 188, 194, 196]. Lesions of the MPOA which include the ventral BnST impair maternal behaviour, and OXT administration to the BnST impairs maternal aggression [56, 67]. However no study has examined the effect of a specific BnST lesion on maternal aggression and the results of the present study plus previous would lead to the hypothesis that a direct lesion of the BnST would significantly attenuate maternal aggression [12, 56, 188, 194, 196].

Fos expression within the PVN, SON and MeA was also significantly lower by 2h following pup removal compared to dams with pups present. The PVN is important in the control of fear and anxiety expression which during pregnancy and lactation is significantly reduced compared to non-maternal female rats; thus this along with the strong positive correlations observed between Fos expression and aggressive behaviour expression adds further evidence to the hypothesis that the reduction of fear and anxiety controlled by this region around the peri-partum period enables maternal aggression [12, 31-34, 188]. The PVN along with the SON is one of the main sources in the brain of OXT, which is significantly linked to maternal aggression control; hence lower Fos activation in the PVN and SON following 2h of pup removal may reflect the lower demand for OXT to drive the maternal aggression circuitry [188, 198, 227, 265, 267]. Microdialysis sampling of the PVN and the SON (chapter 4) in the lactating rat shows an increase in OXT during maternal aggression

expression hence future studies should examine OXT release in the PVN and SON in lactating rats following pup removal during a maternal aggression test to determine if pup cues are essential in contributing to the release of OXT necessary for activation of the maternal aggression circuitry [320]. The MeA is an important brain region in the relay olfactory information so the lower Fos expression after as little as 2h pup removal may be a reflection of the significantly less time spent sniffing the intruder these dams display [70, 401-403]. Indeed there is a significant positive correlation between MeA and sniffing duration. As reduction in Fos activation in these 4 areas (BnST, PVN, MeA and SON) is observed as little as 2h after pup removal it could be proposed that these areas are highly sensitive to pup cues for their actions in maternal aggression regulation.

Fos activation after the maternal aggression test was only significantly lower in the LS and CeA after pup removal for 24h. However correlations of Fos and aggressive behaviour expression did highlight that these regions may be influential in the maternal aggression circuitry (Table 3.1 and appendix 6). The LS has been implicated as being important only in the control of maternal aggression as lesions of the septal region decrease maternal aggression and have no effect on other maternal behaviour [67, 404]. Also, all studies examining IEG activation following a maternal aggression test have observed an increase in activation of the LS [12, 194]. In this study, significant positive correlations were observed for Fos expression within the LS with attack number/latency, attack/biting/clawing/pinning down duration. This may implicate an important role of the LS in maternal aggression but not control as Fos activation in the LS was only lowered once all aggressive behaviour expression had been diminished. The level of Fos expression within the CeA was also

significantly correlated with attack number/latency, attack/pinning down duration thus demonstrating these two areas are also important in the maternal aggression circuitry. The CeA is another brain region important in the control of fear and may link with the PVN and PAG to reduce neophobia and fear of the intruder to enable maternal aggression [105, 405].

Fos expression after the maternal aggression test was only lower after 24h of pup removal within LS and CeA compared to dams with pups present therefore it could be proposed these areas are more sensitive to neuromodulators (such as OXT, GABA, estrogen and progesterone) in their role in maternal aggression control. However, as mentioned above, it could also reflect that they are more involved in enabling maternal aggression rather than its direct control. One way this could be investigated would be to examine Fos expression in rat brains following maternal aggression testing at specific lactation time points. It has been inferred that during the early stages of lactation, maternal aggression is under the influence of the dramatic ovarian hormonal changes postpartum and as these return to normal (i.e. during the later stages of lactation) maternal aggression regulation is more influenced by pup stimulation [56, 181]. Therefore one would expect that areas (BnST, PVN, MeA and SON) where Fos activation after the maternal aggression test was significantly reduced after 2h pup removal there would be no change in expression as the lactation period progresses, but in the other areas (LS, and CeA) Fos expression would be expected to decrease to levels observed in untested lactating rats. Another experiment that would help elucidate whether the hormone and pup cues influence activation of specific brain regions differently for maternal aggression would be to examine Fos expression within virgin female rats, exposed to the maternal

aggression test, who have undergone hormone treatment mimicking pregnancy and parturition. If hormones were important for activation of the LS and CeA, one would expect higher Fos expression in hormone treated virgins compared to vehicle treated non pup-exposed virgins, whereas little or no change would be observed within the BnST, PVN, MeA or SON.

Fos expression within the PAG was only significantly lower after 6h of pup removal but no different after 24h. Thus the involvement of the PAG in the maternal aggression circuitry remains unclear although it is implicated as having a role as significant positive correlations were observed between specific maternal aggression components and the number of Fos positive cells in the PAG. As mentioned in the general introduction the PAG can be divided in different specific regions, each with individual functions hence further research examining the specific areas of the PAG in connection to maternal aggression may be able to more clearly determine the role of the PAG in the maternal aggression circuitry.

Interestingly there was no change in Fos expression observed in the MPOA between dams with pups present or dams whose pups had been removed for 2, 6 or 24h. Furthermore, no significant correlations were observed between the level of Fos expression within the MPOA and display of any specific aggressive behaviour component. The MPOA is the area considered to link the unique circuitries of all maternal behaviour, as MPOA lesions here disrupt all maternal behaviours examined so far [67]. However, studies have yet to examine if the MPOA is critical for maternal aggression, as most lesion studies have only investigated other maternal behaviours [67]. The drawback in investigating the effects of MPOA lesions and maternal aggression is that it would be hard to distinguish whether impairment of

maternal aggression was due to a direct effect on maternal aggression or an indirect one through the disruption of the other maternal behaviours caused by the lesion. Also, although IEG studies highlight that the MPOA is activated after an maternal aggression test, this does reflect how the MPOA may be working in the maternal aggression circuitry i.e. the MPOA may work to inhibit expression of other maternal behaviours allowing the primary output to be maternal aggression [9, 67, 69, 88, 89]. Further research that examines direct infusions of neurotransmitter antagonists to the MPOA which excite or inhibit maternal aggression would demonstrate if the MPOA is essential in maternal aggression as well as most other maternal behaviour. However, the results of our study indicate that the MPOA is more critical for other aspects of maternal behaviour such as nursing, grooming, pup retrieval and nest building and further evidence of this is observed in the pup-sensitized virgin female rats (discussed below).

In the pup-sensitized virgin female rats with pups present, reduced or no aggressive behaviour was observed in comparison to dams with pup present. No attack behaviour was displayed by any pup-sensitized virgin rat; however display of all other aggressive behaviour components was observed. Thus the suite of aggressive behaviours (apart from attack) which make up maternal aggression can be induced partly by stimulation by the presence of pups as observed in previous studies [174]. However these aggressive behaviours are only displayed at levels similar to dams following 24h pup removal and lactation day 21 rats and never at the high levels seen in rats during the first week of lactation, similar to results observed in pup-sensitized ovariectomised rats [174]. It appears therefore that for normal

maternal aggression levels to be expressed in a non-pregnant rat hormonal manipulation is required.

Mayer and Rosenblatt [183] demonstrated that in non-pregnant hysterectomised and ovariectomised rats (to reduce circulating ovarian hormones and cause a switch in the levels of estrogen and progesterone similar to changes at the end of pregnancy), treatment with estrogen could induce aggressive behaviour like maternal aggression even without expression of maternal behaviour (i.e. exposure to pups). Once maternal behaviour was initiated in these rats, no further change was observed in maternal aggression. Yet rats that were estrogen treated following pregnancy termination and then exposed to pups to initiate maternal behaviour, were significantly more likely to exhibit maternal aggression than pregnancy-terminated rats without pup exposure and sensitized non-pregnant sham-hysterectomised and ovariectomised rats [183]. Also, a higher percentage of pregnancy-terminated rats that initiated maternal behaviour displayed maternal aggression than if they were just pregnancy-terminated and estrogen treated [183]. This emphasizes the importance of hormonal influences on the control of maternal aggression and especially the experience of pregnancy hormonal changes (i.e. the rise of progesterone followed by the switch in the estrogen:progesterone ratio at term) for full maternal aggression expression. These hormonal changes together with constant stimulation by pups result in the greatest display of maternal aggression as removal of pups from dams gradually diminishes maternal aggression. It is possible therefore to propose that the importance of hormonal influence is for maternal aggression initiation but possibly not for maternal aggression maintenance. One way to investigate this would be to examine expression of maternal aggression following pup removal after maternal

behaviour initiation in hormonally manipulated pregnancy-terminated rats. If pup stimulation is essential for maternal aggression maintenance and not hormonal influences, one should observe a decrease in maternal aggression similar to that in the study performed in this chapter.

Fos expression was significantly lower in most brain areas (SON, BnST, LS, and CeA) of pup-sensitized virgin rats with pups present following maternal aggression testing compared to dams with pups present. Fos or pCREB expression is also significantly higher in these areas when aggressive mice or rats compared to non aggressive following a maternal aggression test [12, 188, 194]. This indicates the results we observed here are a reflection of the higher aggression displayed by dams compared to virgins. Further evidence of this is provided by the correlation data, where higher Fos expression in these brain regions was positively correlated with higher maternal aggression. Interestingly, in the MPOA, Fos expression appears to be increased following maternal aggression testing in pup-sensitized virgins compared to dams but this is not significant. However, this could be a result of the observed increase in nesting behaviour in pup-sensitized virgins compared to dams with their pups present. Thus, with further research this may show that the MPOA is crucial in the regulation of other components of maternal behaviour but possibly not maternal aggression.

In two brain regions, the PVN and PAG, there was no significant difference in Fos expression between dams and pup-sensitized virgins after the maternal aggression test. As described above these two areas are important in the circuitry for fear and anxiety, which are known to be reduced throughout the peri-partum period [335]. No differences in Fos expression in these areas between dams and pup-

sensitized virgins with pups present during maternal aggression testing adds further evidence that the state of motherhood involves anxiolysis, and that pup cues are important in controlling the actions of these areas on reducing fear and anxiety to allow maternal behaviour. Previous studies have observed that pup-sensitized virgins display reduce anxiety in the EPM and decreased freezing response to auditory noise [42, 174, 406]. They have also shown these changes requires the constant presence of pups, which is supported by the present study as expression of freezing behaviour was significantly higher in virgins after 2h pup removal compared to virgins with pup present [390]. The presence of pups may also be important for the dam's anxiety state to as Fos expression in the PAG and PVN was significantly lower in dams as little as 2h after pup removal although no difference was observed in freezing behaviour (discussed above). Examination of anxiety behaviour before and after pup removal in lactating rats would add further evidence that the presence of pups was important for a dam's reduced anxiety.

Interestingly, there was also no significant difference in Fos expression between virgin pup-sensitised rats with the pups present compared to dams with pups present even though sniffing duration of the intruder was significantly lower. Instead this expression of Fos in the MeA may reflect activation of the MeA by olfactory cues from the pups to induce the expression of maternal behaviour in the virgin rats.

The non-pregnant rat therefore remain a useful tool in advancing our knowledge about how peri-partum hormonal changes and/or cues from pups regulate specific brain areas involved in the neural circuitry of maternal aggression. This is because these rats can be induced by specific hormone manipulation, or exposure to pups without the influence of ovarian hormones (by hysterectomy and ovariectomy),

to express maternal behaviour. Future research using these models examining application of hormones to specific brain regions and their effect on maternal aggression and other elements of maternal behaviour will help define the specific roles of these hormones and peptides in maternal aggression regulation. There is a wide range of hormones and peptides currently implicated as being involved in maternal aggression and maternal behaviour control, from the neuropeptides OXT and AVP, which are investigated and discussed in chapter 1 and 4, to ovarian hormone derivatives such as AP (chapter 1 and 5), to neurotransmitters such as GABA, which was examined in chapter 6.

The main other factors currently being researched in the control of maternal aggression are CRH, prolactin and serotonin [188]. CRH is involved in the HPA axis to activate fear and anxiety; during lactation the HPA axis is hyporesponsive so lower central release of CRH may prevent activation of fear and anxiety behaviour to enable maternal aggression. This is supported by evidence that application of CRH and related peptides, Urocortin 1 and 3, lower maternal aggression in lactating mice [188, 204, 205, 407]. Prolactin, as described in chapter 1, is essential for maternal care but links with maternal aggression are currently unclear, though centrally released prolactin is indicated as possibly being involved [188]. In males, the link between aggression and serotonin is well established with high serotonin levels inhibiting aggression and low level facilitating aggression [188, 378, 408-413]. However, in females the picture is still unclear as elevated serotonin has been correlated with lower maternal aggression, but studies examining the effects of serotonin antagonists have also observed lower maternal aggression in lactating rodents [177, 188, 414-416].

Following a maternal aggression test, aggressive lactating rats display significantly reduced anxiety behaviour. They make more entries into the open arm and spend significantly longer in the open arms compared to non-aggressive lactating rats. However, caution should be applied when interpreting the results because aggressive rats moved much faster on the open arms and travelled a further overall distance. Thus these results may actually reflect an increase in vigilance against intruders, or searching for pups that would be expected during the lactation period. Yet it has been proposed that the EPM is an excellent model to assess state anxiety as demonstrated by extensive research examining the effects of a prior stressful challenge upon anxiety state on the EPM [417]. For example, experience of restraint stress or social defeat prior to placement of the EPM reduces time spent in the open arms, indicating an increased anxious state [418-422]. Therefore it could also been concluded that a lactating rat in an aggressive state shows reduced anxiety. Virgin rats are normally fearful of pups and actively avoid them; however constant exposure to pups will induce expression of maternal behaviour [10, 29]. It also, as discussed above, induces a reduced anxiety state, hence it is the state of motherhood not aggression that reduces anxiety, so the lower anxiety profile on the EPM most likely reflects the increased vigilance of the lactating rat in an aggressive state [174, 406].

To conclude, this chapter has emphasized that maternal aggression is not only reliant on the dramatic hormonal changes of the peri-partum period but for full maternal aggression display it requires cues from pups. It has also demonstrated that the BnST, MeA, PVN and SON brain regions involved in maternal aggression may be more sensitive to pup cues to control maternal aggression than the LS and CeA in lactating rats. Fos expression within the PVN and PAG after maternal aggression

testing was not different between lactating and pup-sensitized virgin rats with pups present, indicating that the state of motherhood, whether hormonal or pup-induced, influences the anxiety and fear circuitry in the brain. Furthermore, we have also demonstrated that following a maternal aggression test the lactating rat exhibits a reduced anxiety profile reflecting increased vigilance for intruders or searching for pups.

Chapter Four: Maternal aggression and the neuropeptides, Oxytocin and Vasopressin.

4.1 Introduction

Maternal aggression as discussed in chapter 1 and 3 is an important behaviour only expressed around the peri-partum period in rats [168, 179, 181]. The peri-partum period is a time where expression of social behaviours, such as attachment and bonding between mother and offspring, are essential to ensure survival. It is therefore unsurprising, research is uncovering substantial evidence for the involvement of OXT and AVP in the regulation of maternal behaviour especially maternal aggression because these two neuropeptide are essential in the control of many different social behaviours [23, 25, 107, 152, 227, 228, 266, 277-279, 284-286, 298-300, 315, 331-334, 345, 423-425].

In late pregnancy, disinhibition of $GABA_A$ receptors just prior to parturition causes a dramatic rise in OXT levels both peripherally and centrally [307]. The peripheral OXT release from the posterior pituitary gland is important to drive parturition and the milk ejection reflex [130, 131, 307, 316]. During parturition, in all mammals (where it has been investigated) higher levels of OXT secretion are observed along with an increase in uterine responsiveness to OXT [316]. This is as a consequence of an increase in OXT receptor (OTR) expression in the uterus, along with increases in uterine contractility, so OXT is a powerful uterotonic agent important for allowing parturition to progress normally [130]. Further evidence of OXT's crucial role in parturition is when an OXT antagonist injected intravenously not only disrupts the onset of birth, but if it is administered after the start of parturition (i.e. after birth of second pup) subsequent pup births will be delayed in

mice [426, 427]. In humans, this powerful effect of OXT antagonist administration means it is now used in clinical practice to prevent preterm labour [427].

However, creation of OXT knock out (KO) mice has recently brought into question how essential these OXT actions on parturition and maternal behaviour are, as these OXT KO mice are able to give birth and express maternal behaviour normally [316]. Therefore it is now considered that there is some redundancy in the need for the effects of OXT on parturition and maternal behaviour [316]. In terms of the milk ejection reflex however, the OXT KO mice further highlight the indispensable role of OXT in controlling this function as they are unable to transfer milk to their young [316]. However, although OXT has been knocked out in these mice, AVP is still present and because of a similar structure to OXT, AVP can act upon OTRs [200]. Thus this ability of OXT KO mice to give birth and express maternal behaviour may be due to activation of OTRs by AVP. Also, as OXT in these KO mice is deleted from birth, it is unknown what the long term effects of the absence of OXT there are on behaviour. Furthermore, OXT expression is absent throughout the brain and OXT may have differing functions in different brain regions where it might inhibit one area but activate another to result in a specific behavioural output. Future research involving OXT KO mice should try to focus on enabling researchers to delete OXT at a specific time point and in specific brain regions to help elucidate more clearly the actions of OXT on maternal behaviour including maternal aggression [428].

Peripheral OXT secretion has clear functions at term in control of parturition and lactation however very little of peripherally secreted OXT is able to cross the blood brain barrier. Hence it is proposed that centrally released OXT is important for

the actions of OXT on maternal behaviour including maternal aggression [318]. It was first hypothesised that OXT was essential for initiation and maintenance of maternal behaviour, as ICV OXT administration to nulliparous ovariectomised rats could induce maternal behaviour whereas infusion of an OXT antagonist ICV in rats was able to prevent maternal behaviour expression [131, 316, 317]. However it was later observed that once maternal behaviour was initiated, OXT antagonist administration had no effect on maternal behaviour [316]. This implies that OXT is a requisite for the onset of maternal behaviour but not its maintenance [316]. Immediately following parturition in the rat, significant increases in OTR mRNA expression is observed in the SON, BnST and MPOA (areas closely linked with maternal behaviour) [9, 67, 80, 82, 83, 90, 148, 188, 307]. These increases are probably a result of the uterine contraction, cervical and vaginal distension at parturition or physical and olfactory and sensory stimuli from pups [318]. OTR expression in these specific brain areas rapidly decreases by 12h postpartum to virgin rat levels; this is further evidence that OXT may only be necessary for maternal behaviour commencement and not its continuation [318].

Research also suggests that OXT plays an important part in the control of maternal aggression, however the literature is conflicting and its precise role is as yet unclear. In rats, administration of antisense oligonucleotide to OXT mRNA (to reduce OXT synthesis) or ibotenic acid (which damages neurons that express glutamate receptors) into the PVN results increased maternal aggression expression whereas electrolytic lesions of the PVN causes a decrease in maternal aggression [269, 429]. The difference between these two studies is hard to reconcile as both were in the same species of rat, surgery was performed at the same time point and

behavioural testing occurred at the same time point [227]. More recently though, it has been observed that OXT release is increased more within the PVN in high anxiety behaviour (HAB) compared to low anxiety behaviour (LAB) rats during a maternal aggression test [320]. Lactating HAB rats are far more aggressive than lactating LAB rats hypothesised to be due to the even higher level of OXT secretion in HAB rats during a maternal aggression test [36, 320, 430]. Furthermore, if an OXT antagonist is administered centrally to a lactating HAB rat maternal aggression is reduced, whereas administration of OXT to a lactating LAB rat will increase levels of maternal aggression [320]. This indicates that OXT within the PVN acts to increase maternal aggression; however disparities still remain and further research needs to be performed to confirm OXT actions in the PVN on maternal aggression.

By contrast, the role of OXT within the CeA is more clearly defined. In the rat, OXT injections directly into the CeA (and also the BnST) decrease maternal aggression [431]. In agreement with this, direct infusion of an OXT antagonist into the CeA increases aggressive behaviour in the lactating rat [432]. In the lactating hamster, however, direct injection of OXT into the CeA increases maternal aggression [268]. These differences in OXT effects on aggression in the CeA could be as a result of a species difference. The CeA is an area known to be a key regulator in the control of fear [265, 266]. OXT transmission within the CeA has been shown to increase the inhibitory GABAergic action on the AVP fear-inducing neurons of the CeA, thereby reducing fear expression [265, 266]. Thus, there is clear evidence for OXT to have an important role in the control of maternal aggression and the main conclusion currently is that OXT works by modulating fear and anxiety to allow the expression of maternal aggression [265].

Whereas the actions of OXT around the peri-partum period are clearly characterised especially on parturition, lactation and maternal behaviour, the role of AVP is still relatively unknown [424]. Even though AVP is closely related to OXT and can act on OTRs, it appears to have little or no effect on parturition or lactation [200, 316, 433]. Despite this, research has observed an increase in AVP mRNA expression in the PVN and significant changes in central AVP secretion around the peri-partum period [297, 330, 424]. Thus these changes and the importance of AVP in social behaviour would lead to the proposal that AVP in the peri-partum period could have a role in maternal behaviour. Infusion of an AVP antagonist into the MPOA inhibits the onset of maternal behaviour but this could be due to the ability of AVP to activate OTRs [434]. More recently though, infusion of an antisense AVP-R oligonucleotide into the MPOA to down regulate AVP receptors resulted in decreased maternal behaviour, specifically reduced frequency of arched back nursing, time the lactating rat spent on pups and impaired pup retrieval. These findings imply a direct role for AVP, within the MPOA, for the regulation of maternal behaviour after its initiation [424]. Interestingly, these changes in AVP receptor expression did not alter the anxiety behaviour expressed on the EPM suggesting that AVP actions on maternal behaviour are independent of its role in stress and anxiety [424]. Although it is important to note these manipulations of AVP-R expression only occurred locally within the MPOA, and there are other areas linked with maternal behaviour, such as the PVN and CeA, where AVP expression and its control over stress and fear are more clearly established [90, 132, 194, 266].

Administration of an AVP V1a receptor antagonist into the MeA of lactating rats disrupted maternal memory providing further evidence that AVP is important in

controlling maternal behaviour; maternal memory is the ability of postpartum rats to express maternal behaviour to foster pups after a period of separation from their own pups [435]. AVP therefore appears to have an important role in the maintenance of maternal behaviour, especially maternal care; however little research has focused on its role in maternal aggression even though AVP is already directly linked with other forms of aggressive behaviour especially in males.

Research has shown that AVP has an important role in intermale aggression in hamsters especially when acting on the anterior hypothalamus (AH) [16, 23, 25, 436]. Social isolation of male hamsters was observed to increase aggression as well as increasing the number of V1a AVP receptors in the AH [436]. A V1a receptor antagonist applied to the AH was able to inhibit aggression [436]. In rats, AVP has been linked with play fighting among juvenile males following maternal separation [23]. Early life stress, modelled in Veenema *et al* (2008, [23]), by separating males from their mothers (termed maternal separation), is a risk factor for later social and behaviour problems. Veenema *et al* (2008, [23]) observed that maternal separation in the first few days of life increased AVP mRNA expression in the PVN as well as offensive play fighting in juvenile males, a precursor for aggressive behaviour later in life. These increases in AVP mRNA in the PVN were also observed in adult male rats and mice along with increased aggressive behaviour suggesting early life experience can determined the ability in later life to deal with social situations [23, 345]. In relation to maternal aggression however, administration of an AVP V1a receptor antagonist to lactating rats was observed to cause an increase in maternal aggression [437]. Ibotenic lesion studies have also observed AVP inhibiting maternal

aggression in lactating rats [269]. This suggests AVP may act in an opposite way to its actions on male aggression and inhibit the expression of maternal aggression.

In this chapter, one aim was to build a clear and detailed picture of the changes in different aggressive behaviours through the peri-partum period by quantifying maternal aggression at specific time points during pregnancy, parturition and lactation. This information can then be used to relate specific hormonal changes to different behavioural changes during the peri-partum period. The second aim was to investigate the hypothesis that OXT and AVP both have regulatory roles in the exhibition of maternal aggression. This is examined in a series of studies. The first study examined the changes in OTR and AVP receptor binding and AVP V1a mRNA receptor expression throughout the peri-partum period and investigated if any changes observed correlate with the variation in the level of maternal aggression expressed from the study above. The second study examined OXT and AVP secretion in specific brain areas during a maternal aggression test at specific time points through the peri-partum period when there is large variance in levels of maternal aggression. The specific time points for groups to be tested were selected from the results of the first study examining maternal aggression through the peri-partum period. The brain areas selected were those which were highlighted as important in the maternal aggression circuitry in previous Fos studies (Meddle *et al* unpublished [12, 194, 196]) and the study examining OXT and AVP binding. Finally, it was investigated if activation of the maternal aggression circuitry results in activation of OXT sensitive cells by examining double labelled Fos and OTR cells in the brains of lactating rats following a maternal aggression test at the time of the highest maternal aggression. The microdialysis, receptor autoradiography and insitu

hybridisation experiments were done in collaboration with Dr Oliver Bosch and his students, Johanna Pfortsch and Steffi Kaha at the University of Regensburg, Germany.

4.2 Maternal aggression through pregnancy, parturition and lactation in the rat

4.2.1 Method

On day 16, 19, 20 or 21 (PD16, n=7; PD19, n=7, PD20, n=7; PD21, n=8) of pregnancy, day of parturition (1h after birth of last pup, PD; n=7) or days 4-7 (LD4-7; n=12), 14 (LD14; n=8), 21 (LD21; n=9) of lactation, SD rats were subjected to a 10 min maternal aggression test followed by immediate decapitation. The brain was carefully removed and frozen on dry ice for storage at -80^{o}C until required for OXT or AVP autoradiography or AVP V1a receptor ISH.

4.2.2 Results

4.2.2.1 Aggressive behaviour

Aggressive behaviour changed dramatically over the peri-partum period. The number of attacks changed significantly, with increases first seen on PD21 ($p<0.001$ vs PD16, PD20 and LD21, $H_7=52.16$; Fig. 4.4) and then the highest display observed on LD4-7 ($p<0.001$ vs PD16, PD19, PD20 and LD21, $H_7=52.16$; Fig. 4.4). The latency to attack showed similar changes with a significantly lower levels observed first on PD21 ($p<0.001$ vs LD 21, $H_7=43.05$) which continue through PD ($p<0.001$ vs PD16 and LD21, $H_7=43.05$) until LD4-7 ($p<0.001$ vs PD16, PD20 and LD21, $H_7=43.05$; Fig. 4.4). Duration of attacks (secs) was significantly higher on PD21 ($p<0.001$ vs PD16, PD20 and LD21, $H_7=51.81$; Fig. 4.2).

Although significant changes are observed in the display of attack behaviour as early as PD21, the amount of time spent expressing all aggressive behaviours is only significantly higher on LD4-7 when LD4-7 aggression tested rats spend a significantly higher percentage of total time displaying aggressive behaviour than PD16, PD20, LD14 and LD21 aggression tested rats ($p<0.001$, $H_7=38.69$; Fig. 4.1). Furthermore, LD4-7 rats spent significantly more time expressing attack ($p<0.001$ vs PD16, PD19, PD20 and LD21, $H_7=51.81$), clawing ($p<0.001$ vs LD21, $H_7=28.03$) and pinning down ($p<0.001$ vs PD16, PD19, PD20, LD14 and LD21, $H_7=39.20$) behaviours (Fig. 4.2). Interestingly, the duration of biting, one specific aggressive behaviour, was significantly higher only on PD ($p<0.001$ vs PD16, PD20 and LD21, $H_7=32.84$) whereas there was no significant difference on LD4-7 compared to any group (Fig. 4.2). The duration of sniffing was significantly higher on LD4-7 ($p<0.001$ vs PD, LD14 and LD21, $F_{(7,58)}=5.08$) whereas it was significantly lower on PD ($p<0.001$ vs PD20 and LD4-7, $F_{(7,58)}=5.08$; Fig. 4.4).

There were no significant differences in the duration of rearing ($p>0.05$, $H_7=21.30$) or lunging ($p>0.05$, $H_7=22.67$) behaviour throughout the peri-partum period (Fig. 4.2).

4.2.2.2 Maternal behaviour

Maternal behaviour was significantly higher on PD compared to LD4-7 and LD21 ($p<0.001$, $H_3=21.22$; Fig. 4.1 and 4.4). This included a significant increase in the time spent nesting and nursing compared to all lactating tested groups ($p<0.001$ vs LD4-7, LD14 and LD21, $H_3=23.46$).

4.2.2.3 General behaviour

Duration of general behaviours was significantly lower on PD ($p<0.001$, $F_{(7,58)}=19.22$) and LD4-7 ($p<0.001$, $F_{(7,58)}=19.22$) compared to all other groups (Fig. 4.1). There was significantly greater time spent displaying general behaviour on LD21 compared to LD14 ($p<0.001$, $F_{(7,58)}=19.22$; Fig. 4.1). A significant decrease in time spent exploring was observed in the PD ($p<0.001$ vs PD16, PD19, LD14 and LD21, $H_7=39.64$) and LD4-7 ($p<0.001$ vs PD16, PD19 and LD21, $H_7=39.64$) but no significant difference was observed in grooming self behaviour expression across all groups ($p=0.058$, $H_7=13.63$; Fig. 4.3).

4.2.2.4 Response to aggression behaviour by the resident

There was no significant change by resident rats in the duration of response to aggression from the intruder across the peri-partum period ($p>0.05$, $H_7=16.55$; Fig. 4.1).

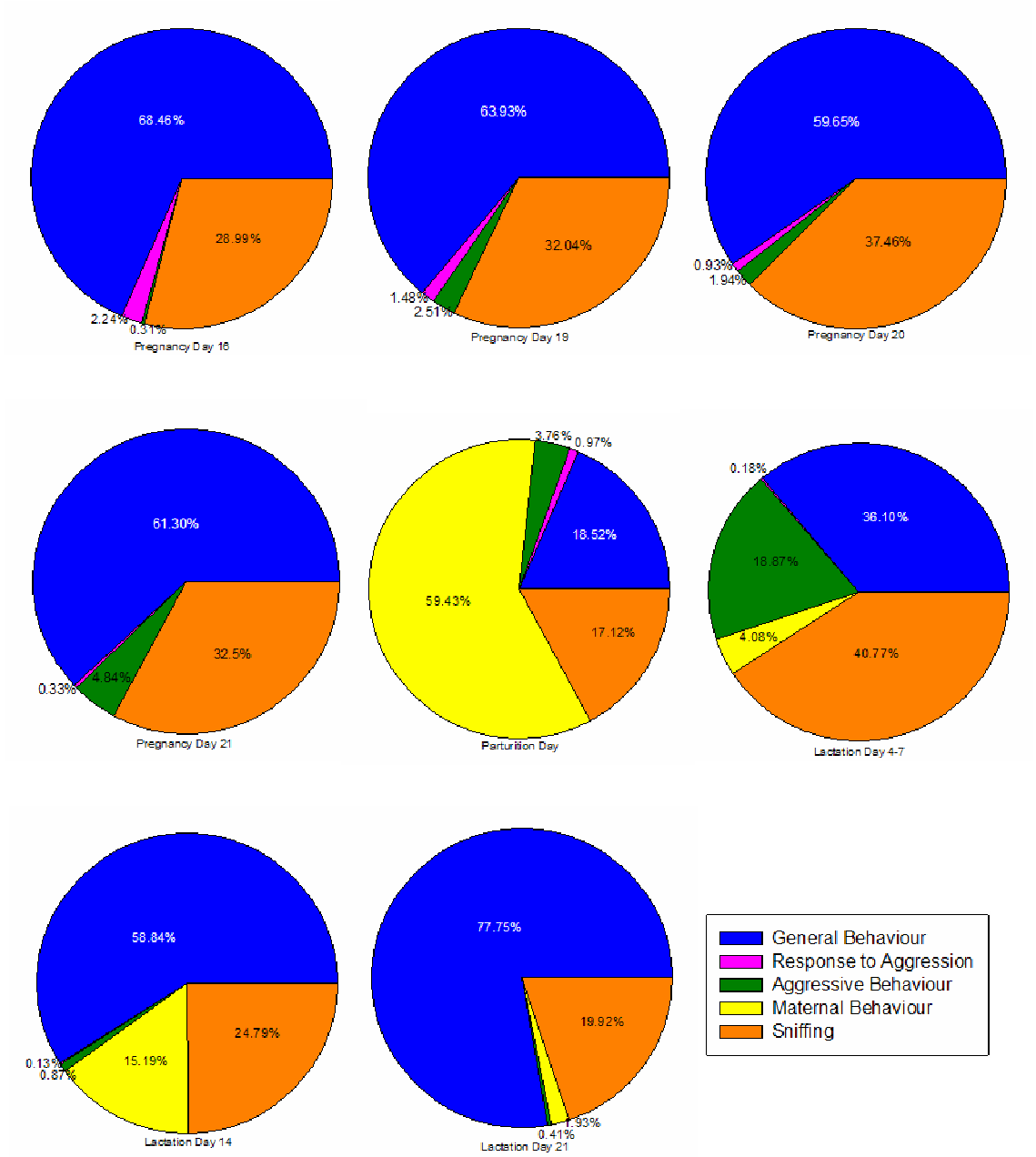


Figure 4.1: Average percentage of total time spent exhibiting different behaviours by the resident rat during a maternal aggression test. The average percentage of the total time of the 10 min maternal aggression test spent by the resident rat (pregnancy day 16, n=7; pregnancy day 19, n=7; pregnancy day 20, n=7; pregnancy day 21, n=8; day of parturition [PD], n=7; lactation day 4-7 [LD4-7], n=12; lactation day 14 [LD14], n=8 and lactation day 21 [LD21], n=9) exhibiting aggressive (including attacks, bites, lunging), maternal (including pup moving and nursing), response to aggression (including freezing) and general (including exploring, eating and drinking) behaviours. The rats in the parturition day group were tested 1h after the birth of the final pup. For PD, LD 4-7, LD14 and LD21, the pups were present during the maternal aggression test. One should note that towards the end of pregnancy aggressive behaviour starts to increase. This level of aggression remains on the day of parturition even with the demand for an increase in maternal behaviour. The peak in aggressive behaviour display occurs during the first week of lactation and is diminished by lactation day 21.

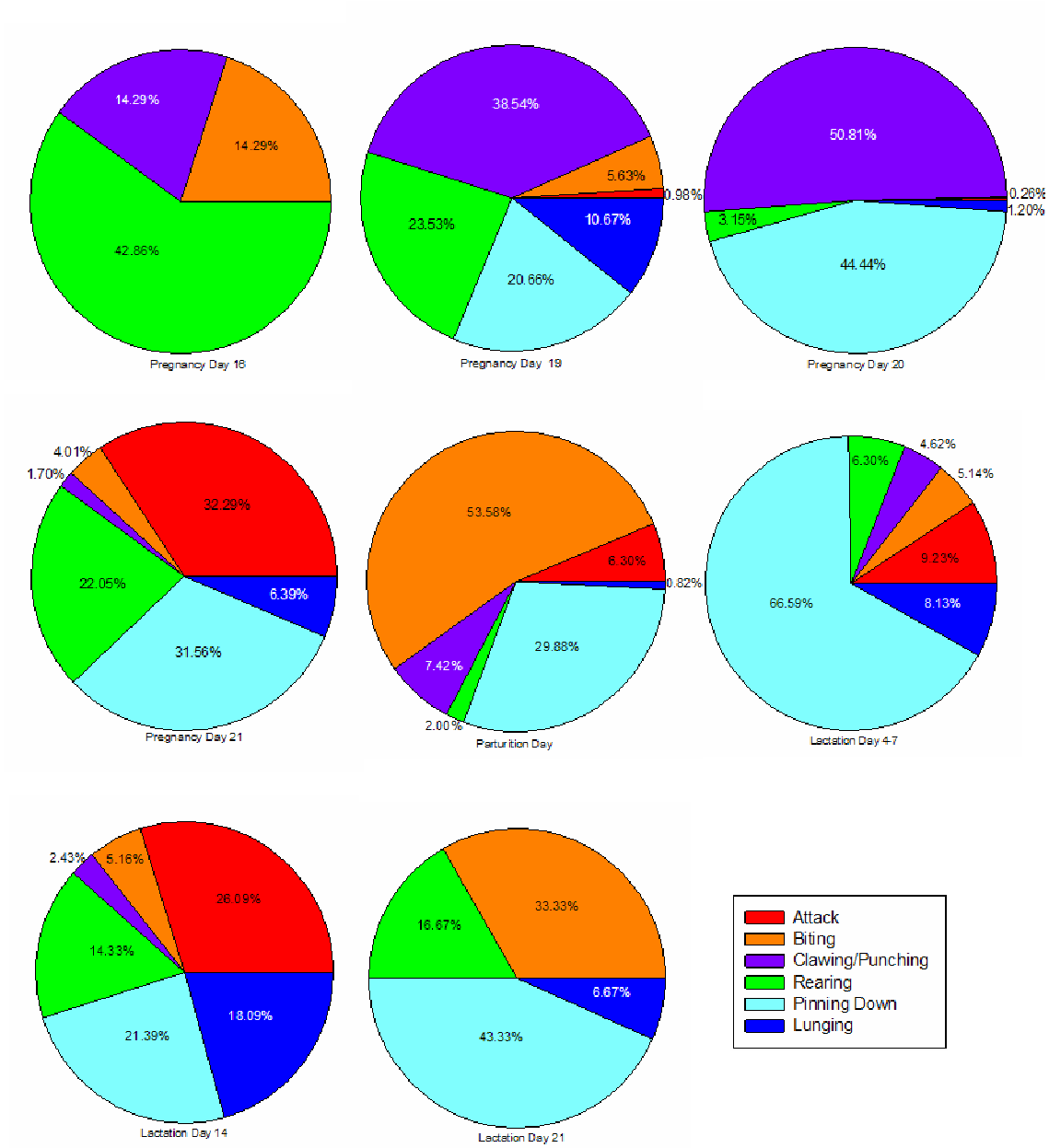


Figure 4.2: Average percentage of total aggression time spent exhibiting different aggressive behaviours by the resident rat during a maternal aggression test. The average percentage of the total aggression time spent by the resident rat (pregnancy day 16, n=7; pregnancy day 19, n=7; pregnancy day 20, n=7; pregnancy day 21, n=8; day of parturition [PD], n=7; lactation day 4-7 [LD4-7], n=12; lactation day 14 [LD14], n=8 and lactation day 21 [LD21], n=9) exhibiting attacking, biting, clawing/punching, rearing, pinning down, lunging or sniffing behaviours towards a novel female intruder during a 10 min maternal aggression test. The rats in the parturition day group were tested 1h after the birth of the final pup. For PD, LD 4-7, LD14 and LD21, the pups were present during the maternal aggression test. One should note that attack behaviour significantly is only first seen on pregnancy day 21. The pie charts also highlight that specific aggressive behaviours are exhibited at specific time points, on parturition day biting behaviour is mostly expressed whereas during the first week of lactation pinning down is aggressive behaviour expressed the most.

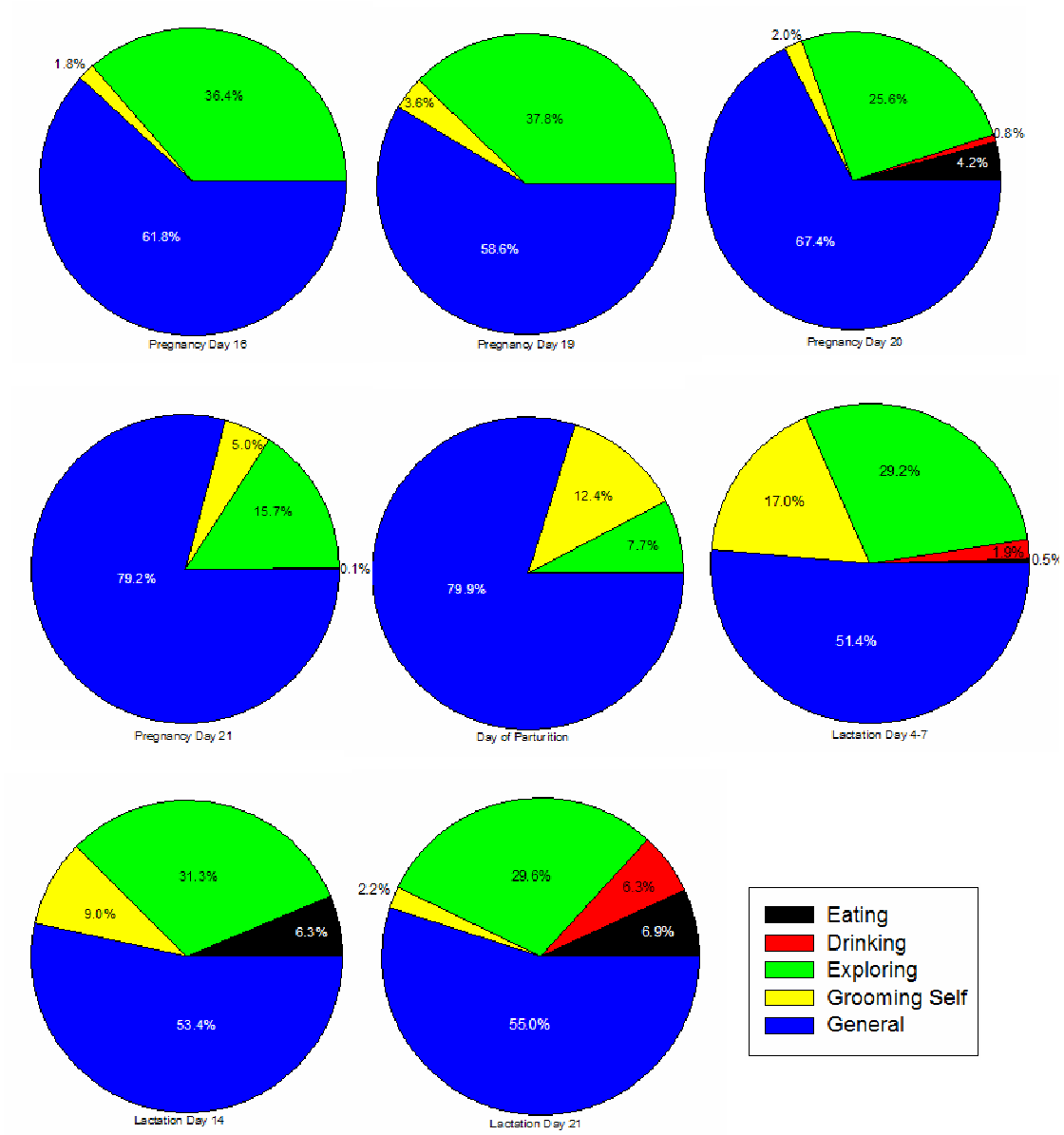


Figure 4.3: Average percentage of total general time spent exhibiting different general behaviours by the resident rat during a maternal aggression test. The average percentage of the total general time the female lactating resident rat (pregnancy day 16, n=7; pregnancy day 19, n=7; pregnancy day 20, n=7;pregnancy day 21, n=8; parturition [PD], n=7; lactation day 4-7 [LD4-7], n=12; lactation day 14 [LD14], n=8 and lactation day 21 [LD21], n=9) exhibited eating, drinking, exploring, grooming self or general (defined as wandering around cage) behaviours during a maternal aggression test. The rats in the parturition day group were tested 1h after the birth of the final pup. For PD, LD 4-7, LD14 and LD21, the pups were present during the maternal aggression test. The important feature to note is the change in time spent self grooming over the peri-partum period.

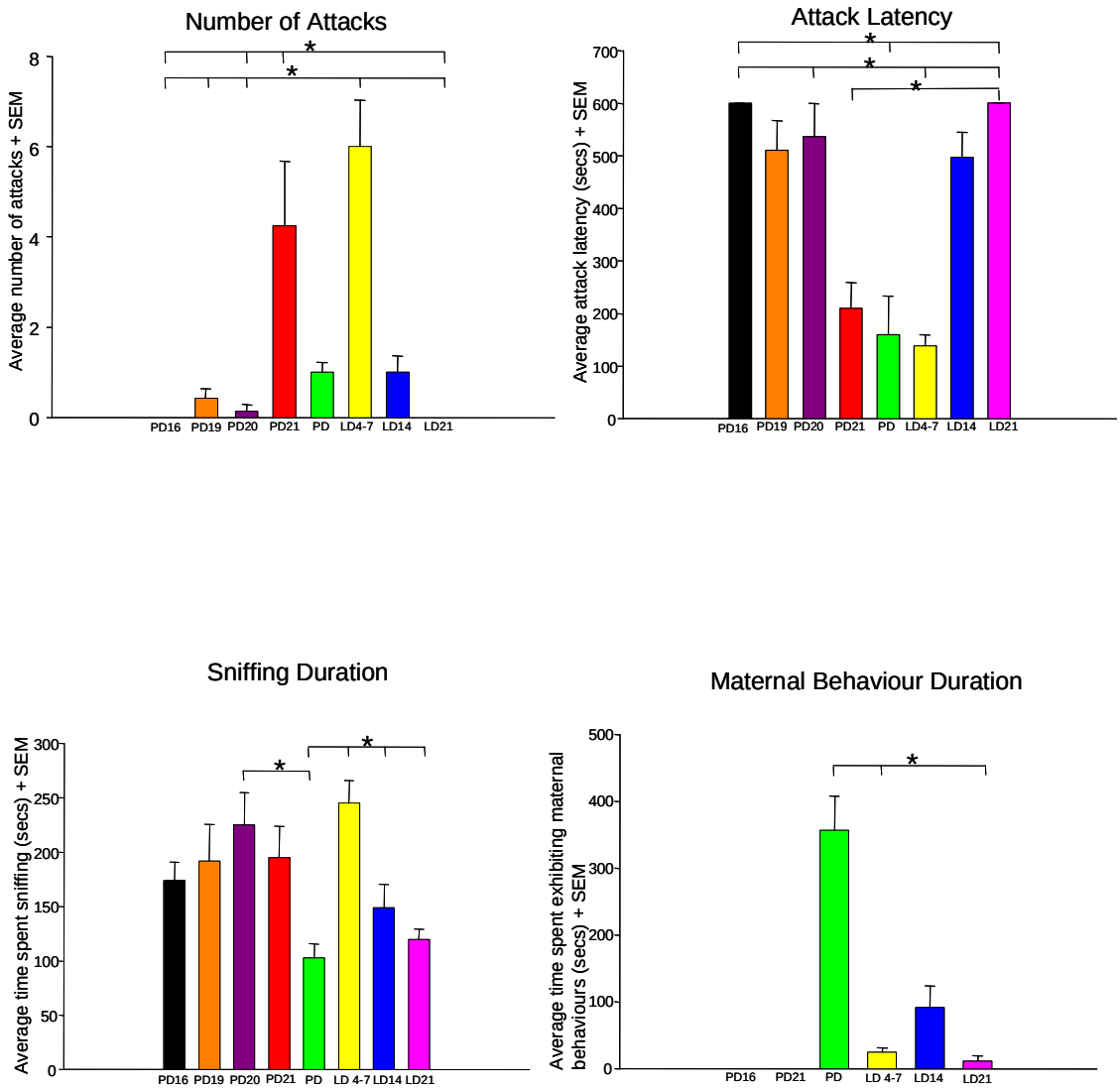


Figure 4.4: Average number of attacks, latency to attack sniffing and maternal behaviour duration during a maternal aggression test by the resident rat. A) The average number of attacks during a 10 min maternal aggression test by the resident rat (pregnancy day 16 (PD16), n=7; pregnancy day 19 (PD19), n=7; pregnancy day 20 (PD20), n=7; pregnancy day 21 (PD21), n=8; day of parturition (PD), n=7; lactation day 4-7 (LD4-7), n=12; lactation day 14 (LD14), n=8 and lactation day 21 (LD21), n=9) in her home cage towards a novel virgin female intruder rat. Rats tested in the PD group performed the test 1h after birth of the last pup. For rats in PD, LD4-7, LD14 and LD21 groups, they were tested with their pups still present. B) The average latency (secs) for a resident rat to attack a novel virgin intruder. The average time (secs) a resident spent sniffing a novel female intruder (C) or exhibiting maternal behaviour (D) during a maternal aggression test. Data are presented as mean ± SEM.

4.3 Oxytocin binding throughout the peri-partum period: comparison to aggressive behaviour

4.3.1 Method

Brains which had been collected from the rats in the first experiment of chapter 4 were serially sectioned using a cryostat (for details see chapter 2) and then processed and quantified for OXT autoradiography in the LS, BnST, MPOA, SON, MeA and CeA using the method described in chapter 2. Films were left to expose in the dark room for 6 days before being developed.

4.3.1.1 Statistics

A one way ANOVA was used to compare the groups in each experiment. If data was not normally distributed a Kruskal-Wallis One Way Analysis of Variance on Ranks was performed. For analysis pregnancy day 16, 19 and 20 groups were combined as there little or no difference in behaviour expressed during the 10 min maternal aggression test (see results of first experiment above). Data was deemed statistically significant when $p \leq 0.05$.

4.3.2 Results

4.3.2.1 Oxytocin receptor binding

OTR binding was significantly higher in the LS of the lactation day 4-7 (LD4-7) group compared to the day of parturition (PD; $p=0.042$, $F_{(5,85)}=2.96$), lactation day 14 (LD14; $p=0.031$, $F_{(5,85)}=2.96$) and 21 (LD21; $p=0.018$, $F_{(5,85)}=2.96$) groups (Fig. 4.5). No difference on OTR binding in the LS was observed between the pregnancy day 16/19/20 (PD16/19/20; $p=0.053$, $F_{(5,85)}=2.96$) or 21 (PD21; $p=0.067$, $F_{(5,85)}=2.96$) group and the LD4-7 group. Within the BnST, OTR binding significantly increased in the PD21 group compared to the LD21 group ($p=0.014$, $F_{(5,88)}=3.19$; Fig. 4.5).

There was no significant difference between any other groups ($p>0.05$, $F_{(5,88)}=3.19$; Fig. 4.5). Interestingly, in the MPOA there was a significant increase in OTR binding in the PD group compared to the LD14 ($p=0.016$, $F_{(5,86)}=3.61$) and LD21 ($p=0.006$, $F_{(5,86)}=3.61$) groups; no difference was observed with between other group ($p>0.05$, $F_{(5,86)}=3.61$; Fig. 4.5). For the PD16/19/20, PD21 and PD groups, there were not enough SON areas per rat to be able to compare OTR binding. There was no significant difference in OTR binding observed in the SON of the lactation day groups ($p=0.40$, $F_{(2,19)}=0.97$; Fig. 4.5). No statistical difference was also observed in the MeA ($p<0.05$, $F_{(5,84)}=3.15$) and CeA ($p=0.382$, $H_5=5.29$) for OTR binding between all groups (Fig. 4.5).

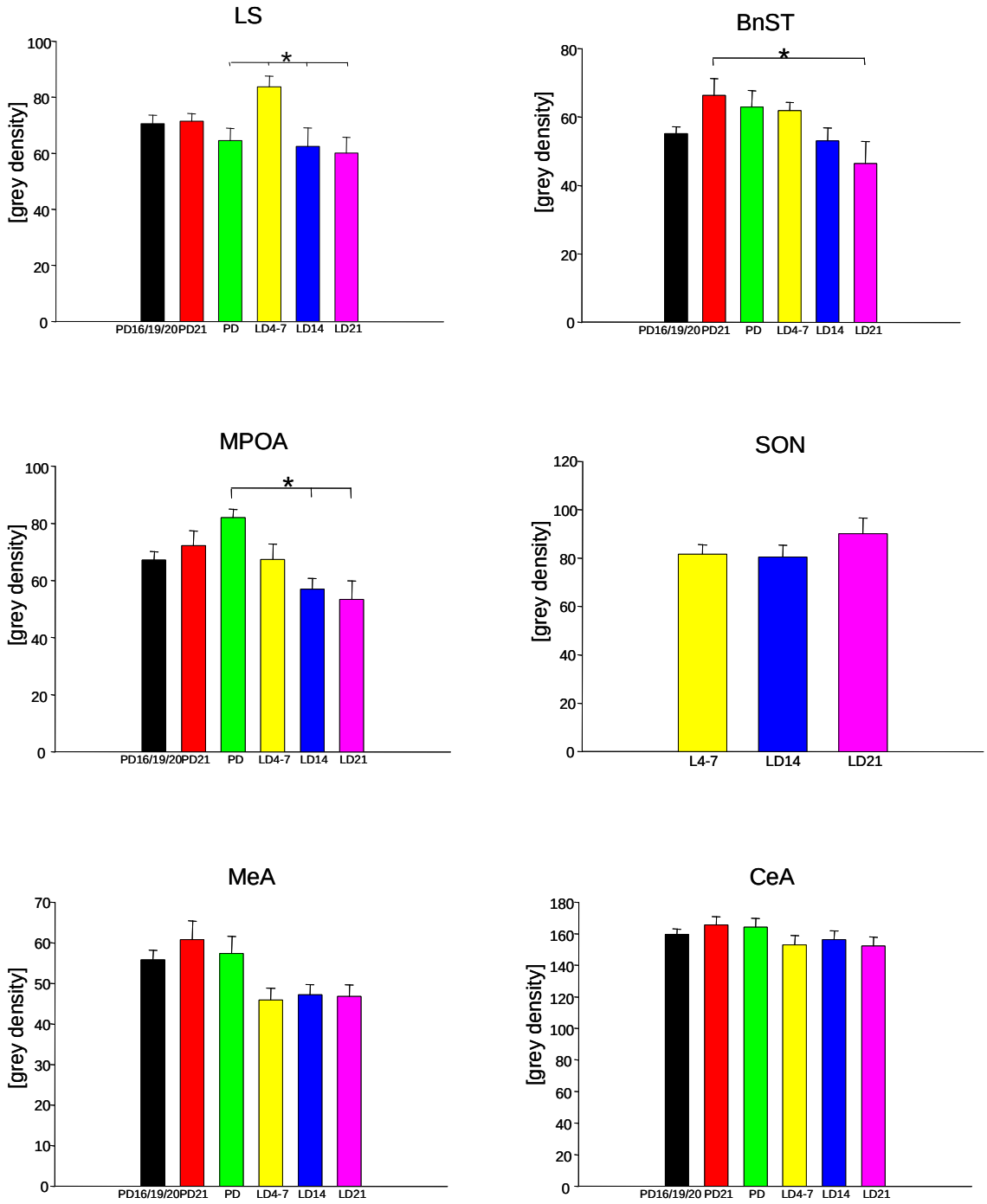


Figure 4.5: Average oxytocin receptor binding in specific brain regions of rats through pregnancy, parturition and lactation following a maternal aggression test. All rats performed a 10 min maternal aggression test followed by immediate decapitation. Brains were collected and frozen on dry ice before being processed for oxytocin receptor (OTR) binding using receptor autoradiography. OTR binding was examined in the lateral septum (LS), bed nucleus of stria terminalis (BnST), medial preoptic area (MPOA), supraoptic nucleus (SON), medial and central amygdala (MeA and CeA respectively) of rats tested on pregnancy day 16, 19, 20 (PD16/19/20, n=21) or 21 (PD21 n=7), day of parturition (PD, 1h after birth of last pup; n=7) or lactation day 4-7 (LD4-7, n=6), 14 (LD14, n=7) and 21(LD21, n=8). Data are represented as mean +SEM. *=p<0.05

4.4 Correlation of maternal aggression with central oxytocin secretion

4.4.1 Method

All rats, pregnant and lactating, underwent bilateral microdialysis cannulation surgery two days prior to behavioural microdialysis test (for details of method please refer to chapter 2). Pregnant rats performed a maternal aggression test on either pregnancy day 19 or 21 (PD19 or PD21). A third group was allowed to continue until full term and tested on lactation day 4 (LD4). Microdialysis cannulas were placed into the BnST (PD19, n=8; PD21, n=7; LD4, n=8), PVN (PD19, n=8; PD21, n=6) LS (PD19, n=8; PD21, n=9) or SON (LD4, n=8) brain regions. One day after testing, all rats were euthanized and their brains collected and frozen on dry ice. Brains were then serially cut on a cryostat and the microdialysis probe placement determined.

The collected microdialysates were sent to Prof Rainer Landgraf at the Max Planck Institute and analysed for OXT levels by radioimmunoassay.

4.4.1.1 Statistics

A one way ANOVA was used to compare behavioural data between groups. For microdialysis, samples 1 and 2 for each group were averaged and defined as 100% basal value. The percentage change in OXT (or AVP) of all samples from the 100% basal value were calculated and used in further analysis. A one way repeated measures ANOVA was used to compare values for samples across a single group. If data was not normally distributed a Friedman Repeated Measures Analysis of Variance on Ranks test was performed. A change in the release of OXT (or AVP) was defined as significant when sample 3 was statistically different from sample 2. A t-test or one way ANOVA was used to compare the sample from different groups collected during the maternal aggression test (i.e. sample 3). If data was not normally

distributed then a Mann-Whitney Rank Sum Test or Friedman Analysis of Variance on Ranks test was performed. When $p \leq 0.05$, data was defined as statistically significant.

4.4.2 Results

4.4.2.1 Behaviour during maternal aggression test

LD4 rats spent significantly longer time sniffing the ano-genital region of the intruder rat compared to the PD19 ($p<0.001$, $H_2=22.96$) and PD21 groups ($p<0.001$, $H_2=22.96$; Fig. 4.6). Less time was spent exploring in the LD4 group compared to the PD19 ($p<0.001$, $H_2=20.73$) and PD21 ($p<0.001$, $H_2=20.73$; Fig. 4.6) groups. No significant difference was observed in number of attacks ($p>0.05$, $H_2=7.30$) or time spent self grooming ($p>0.05$, $H_2=7.14$) between any groups (Fig. 4.6).

4.4.2.2 Oxytocin secretion

OXT secretion within the PVN was found to significantly increase during a maternal aggression test for both PD19 ($p=0.001$, $x^2=17.69$) and PD21 groups ($p=0.05$, $x^2=9.47$; Fig. 4.8). A significant in the release of OXT during a maternal aggression test was also observed within the BnST of the PD19 ($p=0.004$, $x^2=15.43$) and LD4-7 ($p=0.002$, $F_{(7,28)}=8.70$) groups; it was not found to significantly change in the PD21 group ($p=0.08$, $x^2=8.34$; Fig. 4.7). OXT release was also observed in the SON for the LD4-7 where a significant increase was observed during the maternal aggression test ($p=0.002$, $x^2=16.50$; Fig. 4.8). For PD19 ($p=0.21$, $x^2=5.90$) and PD21 ($p>0.05$, $F_{(8,32)}=5.48$) no significant change in OXT release within the LS was observed during the maternal aggression test (Fig. 4.7). There was no significant difference in OXT secretion during the maternal aggression test between PD19 and PD21 groups in the PVN ($p=0.95$, $T_{(6,9)}=49.0$; Fig. 4.8) or LS ($p=0.21$, $t_{-1.31}$; Fig. 4.7).

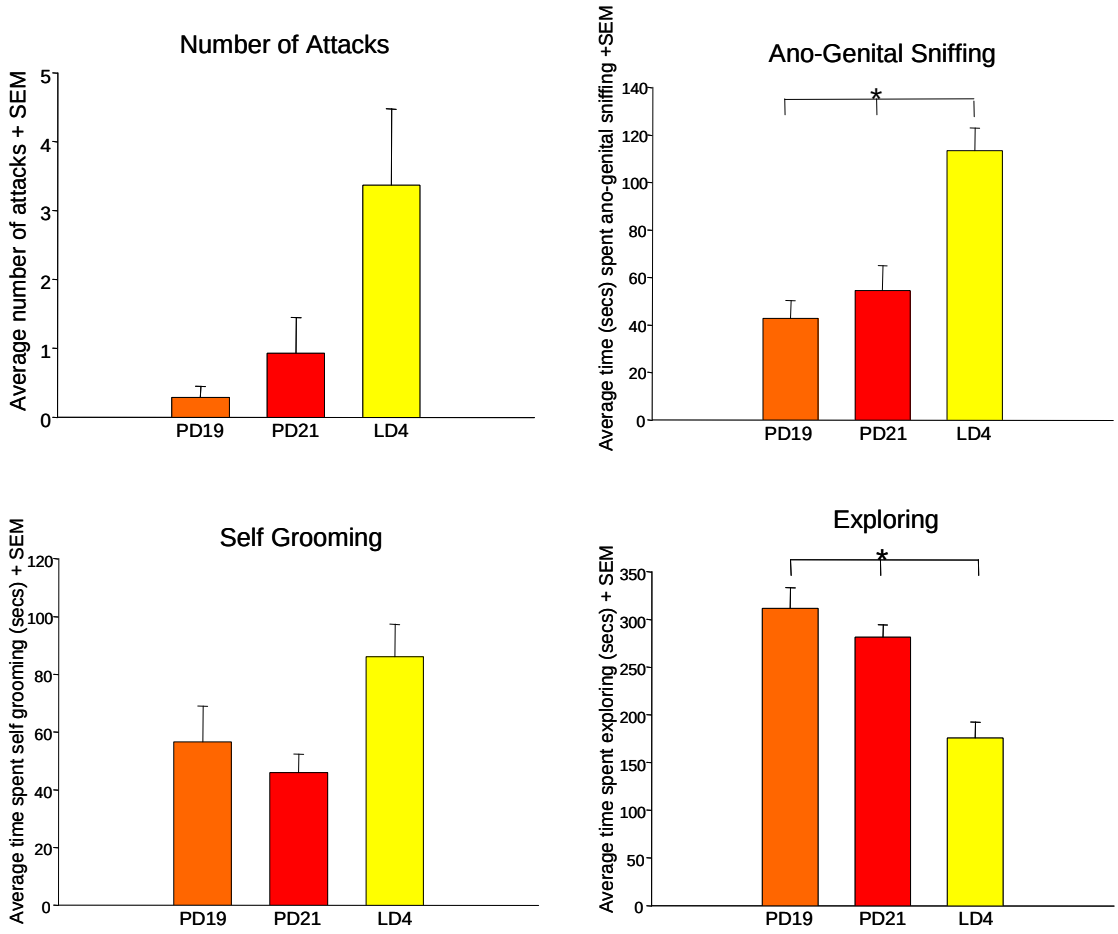


Figure 4.6: Average number of attacks, time spent sniffing, self grooming and exploring for the resident rat during a maternal aggression test. Bilateral microdialysis surgery was performed two days prior to maternal aggression testing on all pregnant and lactating rats. On the day of testing, rats were connected to microdialysis pumps and left to acclimatize for 2h, five dialysates were then collected every half an hour. The first two were collected whilst the rat was left undisturbed in her home cage. The third sample was collected during 10 min maternal aggression test whilst behaviour was digitally recorded. The final two samples were collected with the rat again left undisturbed in her home cage. All dialysates once collected were immediately frozen on dry ice until quantification for oxytocin by radioimmunoassay. The average number of attacks or time spent exhibiting sniffing, self grooming or exploring behaviours during the 10 min maternal aggression test for resident rats (pregnancy day 19 [PD19 n=14] or 21 [PD21 n=14] and lactation day 4 [LD4 n=19]). *p≤0.05. Data are represented as mean + SEM.

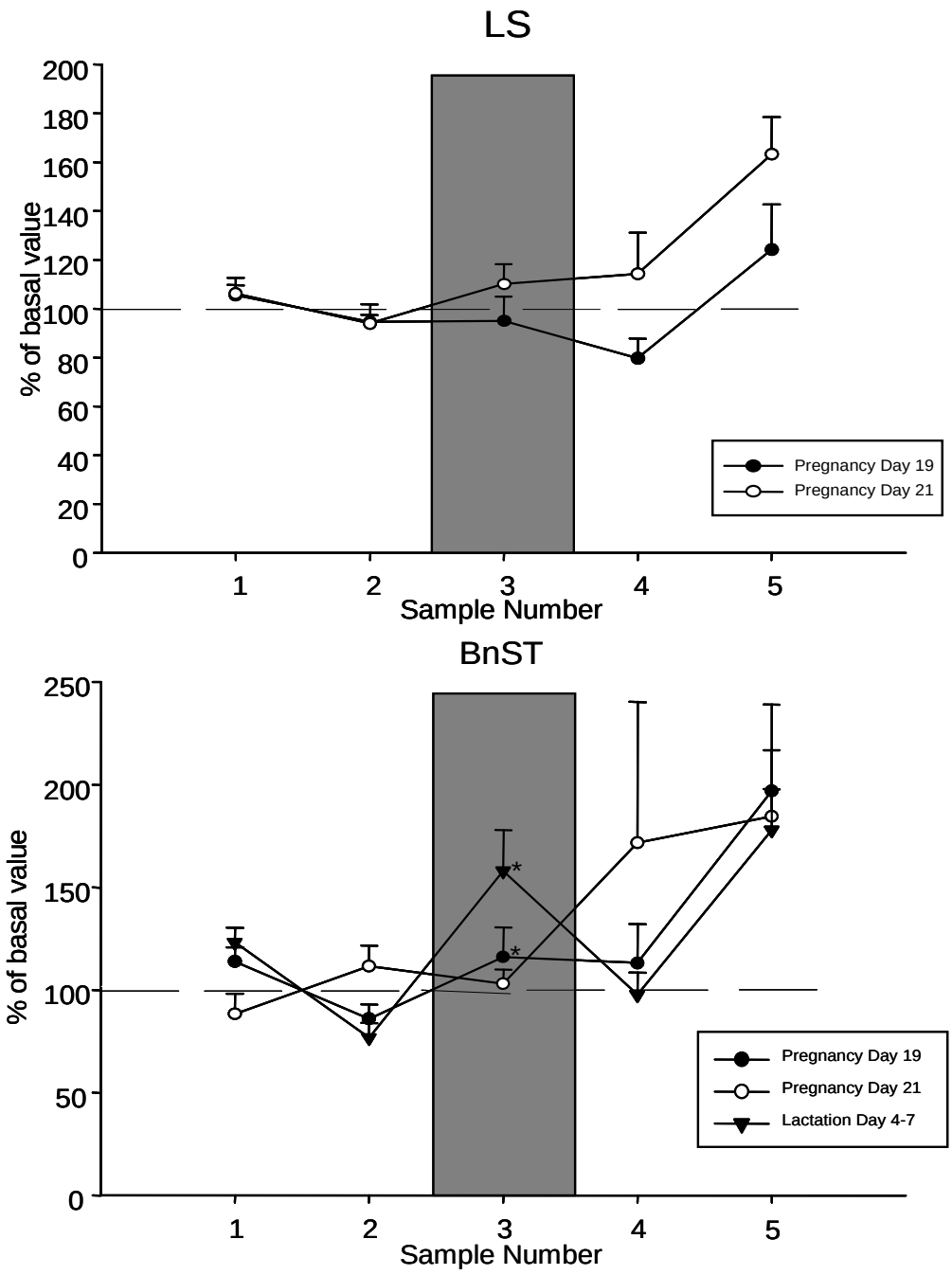


Figure 4.7: Oxytocin release within the lateral septum and bed nucleus of stria terminalis of the resident rat during a maternal aggression test. Bilateral microdialysis surgery was performed two days prior to maternal aggression testing on all pregnant and lactating rats. On the day of testing (pregnancy day 19 [PD19] or 21 [PD21] or lactation day 4 [LD4]), rats were connected to microdialysis pumps and left to acclimatize for 2h, five dialysates were then collected every half an hour. The first two were collected whilst the rat was left undisturbed in her home cage. The third sample was collected during a 10 min maternal aggression test. The final two samples were collected with the rat again left undisturbed in her home cage. All dialysates once collected were immediately frozen on dry ice until quantification for oxytocin by radioimmunoassay. Microdialysis probes were implanted into the lateral septum (LS; PD19 n=8, PD21 n=9) or bed nucleus of stria terminalis (BnST; PD19 n=8, PD21 n=7, LD4 n=8). *=p≤0.05 when sample 3 compared to sample 2. Data are represented mean + SEM.

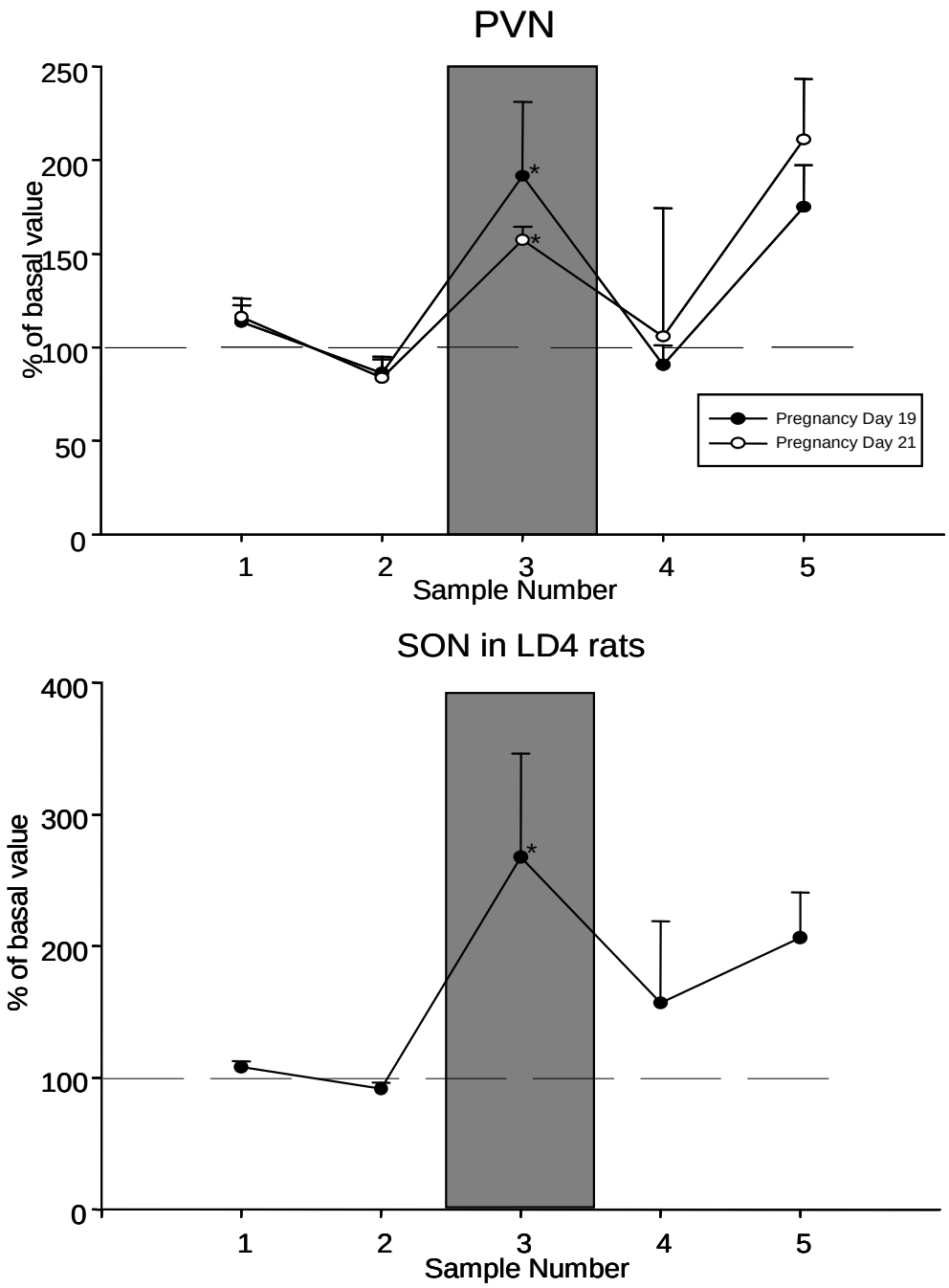


Figure 4.8: Oxytocin release within the paraventricular nucleus and supraoptic nucleus of the resident rat during a maternal aggression test. Bilateral microdialysis surgery was performed two days prior to maternal aggression testing on all pregnant and lactating rats. On the day of testing (pregnancy day 19 [PD19] or 21 [PD21] or lactation day 4 [LD4]), rats were connected to microdialysis pumps and left to acclimatize for 2h, five dialysates were then collected every half an hour. The first two were collected whilst the rat was left undisturbed in her home cage. The third sample was collected during a 10 min maternal aggression test. The final two samples were collected with the rat again left undisturbed in her home cage. All dialysates once collected were immediately frozen on dry ice until quantification for oxytocin by radioimmunoassay. Microdialysis probes were implanted into the paraventricular nucleus (PVN; PD19 n=9, PD21 n=6) or supraoptic nucleus (SON; LD4 n=8). *=p≤0.05 when sample 3 compared to sample 2. Data are represented mean + SEM.

4.5 Mapping maternal aggression neural circuitry containing oxytocin sensitive cells

4.5.1 Method

Between lactation days 3 to 7, rats either performed a 30 min maternal aggression test (n=9) or were left undisturbed (n=8) in their home cage with their pups present (8-18). Lactating rats were perfused 90 min after the start of the maternal aggression test. The behaviour of aggression tested rats was digitally recorded and later analysed to ensure aggression was displayed at levels observed in previous experiments. The brains were collected and processed for Fos and OTR ICC. Fos and OTR positive cells were counted and quantified in the LS, BnST, MPOA, SON, MeA, CeA, PVN, PAG, OBs and VMH, brain areas previously linked with maternal aggression, using the technique described in chapter 2.

4.5.1.1 Statistics

A t-test was performed to compare data between the aggression and non aggression tested lactating rats. Data was deemed to be significantly different when $p \leq 0.05$.

4.5.2 Results

4.5.2.1 Aggressive behaviour

Aggressive behaviour in aggression tested rats was comparable to levels observed in vehicle treated lactating rats from the AP and maternal behaviour study (chapter 5). The average latency to attack was 194.0±63.6 secs (vehicle group for AP and maternal aggression experiment was 167.1±38.8; $p=0.86$, $T_{(9,9)}=88.0$) and average number of attacks was 10.0±2.3 (vehicle group for AP and maternal aggression experiment was 8.2±1.7; $p=0.57$, $T_{(9,9)}=92.5$).

4.5.2.2 Activated oxytocin sensitive cells expression in aggression tested lactating rats

The average number of double labelled Fos and OTR positive cells was significantly higher in aggressive lactating rats in the LS ($p<0.001$, $T_{(8,9)}=36.0$; NAT=21.01±4.8, AT=46.21±12.8; Fig. 4.9 and 4.11), BnST ($p<0.001$, $T_{(8,9)}=37.0$; NAT=13.98±3.7, AT=18.97±2.7; Fig. 4.9 and 4.10) and the MeA ($p=0.001$, $T_{(8,9)}=37.5$; NAT=7.13±1.1, AT= 18.82±2.6; Fig. 4.9 and 4.12) compared with non aggressive lactating rats (Fig. 4.9).

There was no significant difference in the number of double labelled Fos and OTR positive cells in the CeA ($p=0.96$, $T_{(8,9)}=73.0$; NAT=19.59±3.9, AT=19.21±4.8), PAG ($p=0.70$, $t_{15}=0.39$; NAT=56.10±7.4, AT=52.00±7.3), SON ($p=0.74$, $t_{-0.34}$; NAT=12.42±1.3, AT=13.03±1.2), OBs ($p=0.60$, $t_{0.54}$; NAT=113.30±5.9, AT=108.71±6.2), Parvocellular PVN ($p=0.09$, $T_{(8,9)}=90.0$; NAT=11.59±0.8, AT=10.16±0.8), Magnocellular PVN ($p=0.91$, $t_{-0.11}$; NAT=18.48±2.5, AT=18.90±2.8) or MPOA ($p=0.57$, $T_{(8,9)}=74.0$; NAT=86.55±10.0, AT=82.88±11.6) between aggressive and non aggressive lactating rats (Fig. 4.9).

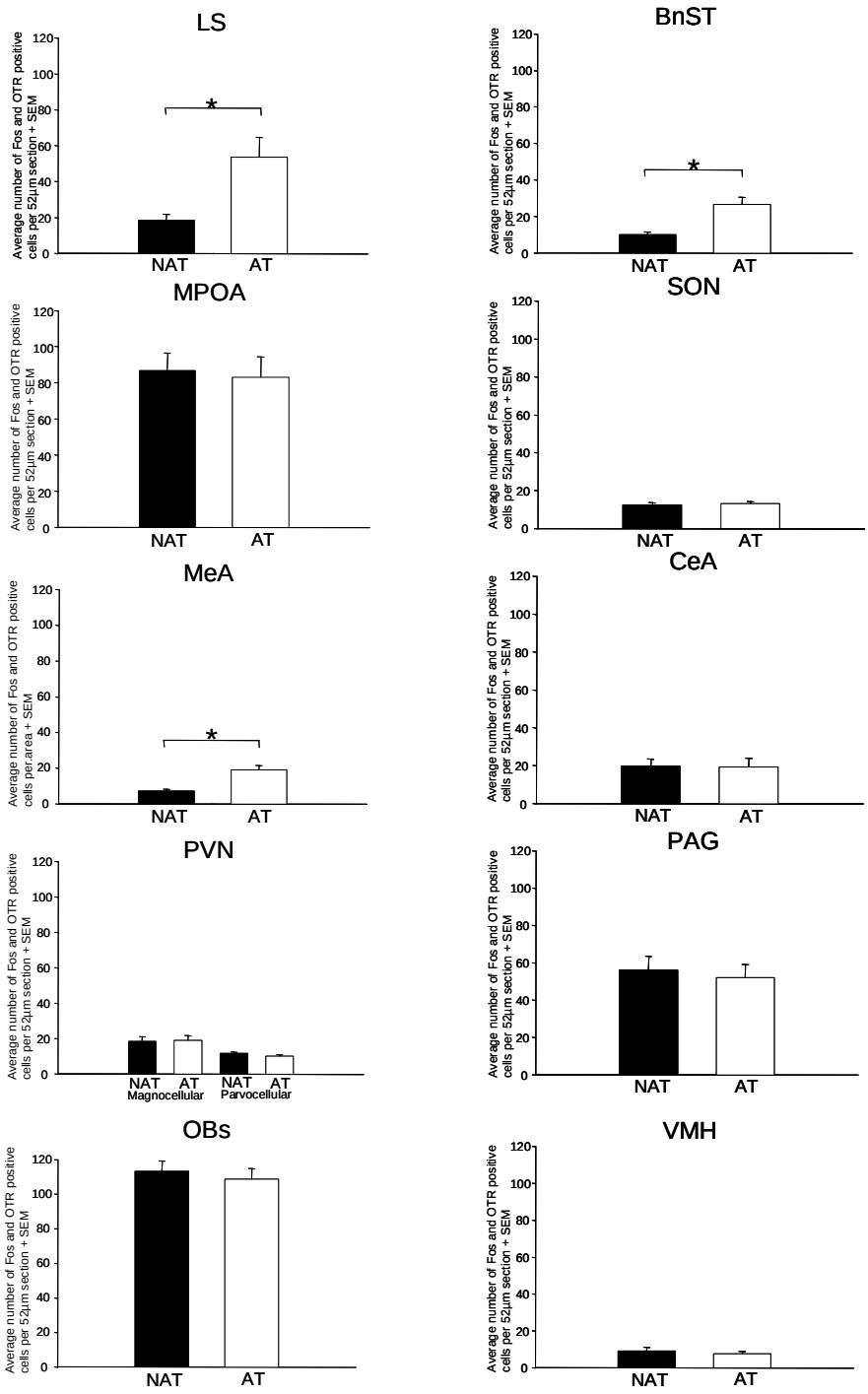


Figure 4.9: Average number of Fos and oxytocin receptor double labelled cells in specific brain regions of lactating rats following a maternal aggression test. Lactating rats either performed a 30 min maternal aggression test or were left undisturbed in their home cage with their pups present, 90 min after the start of testing lactating rats were perfused and their brains collected for immunocytochemistry. Expression of Fos and OTR positive cells was examined in the lateral septum (LS), bed nucleus of stria terminalis (BnST), medial preoptic area (MPOA), supraoptic nucleus (SON), medial and central amygdala (MeA and CeA respectively), paraventricular nucleus (PVN; magnocellular and parvocellular), periaqueductal grey area (PAG), olfactory bulbs (OBs) and ventromedial hypothalamus (VMH) of the aggression (AT; n=9) and non aggression (NAT; n=8) tested lactating rats. Data are represented as mean +SEM. *=p<0.05

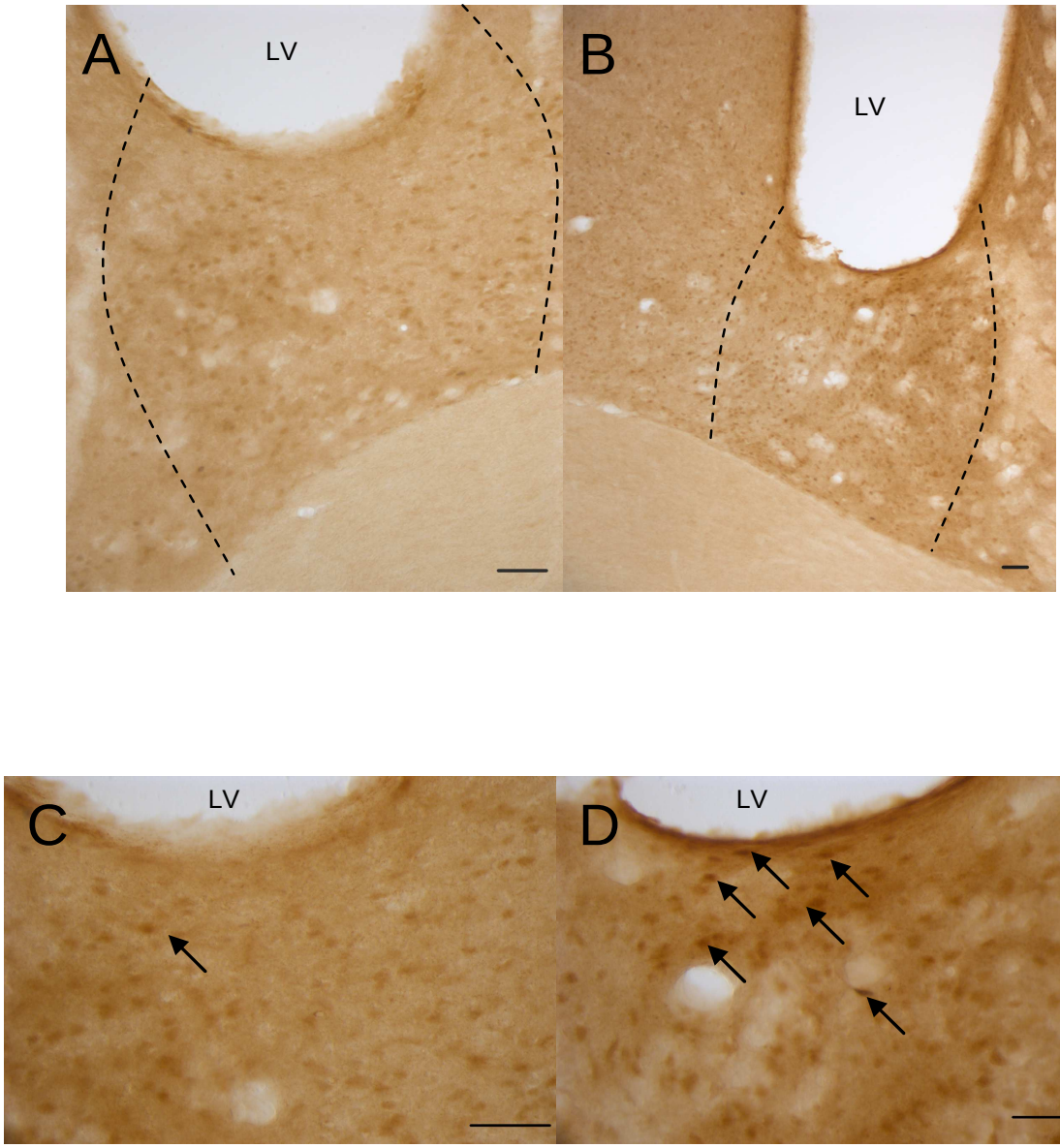
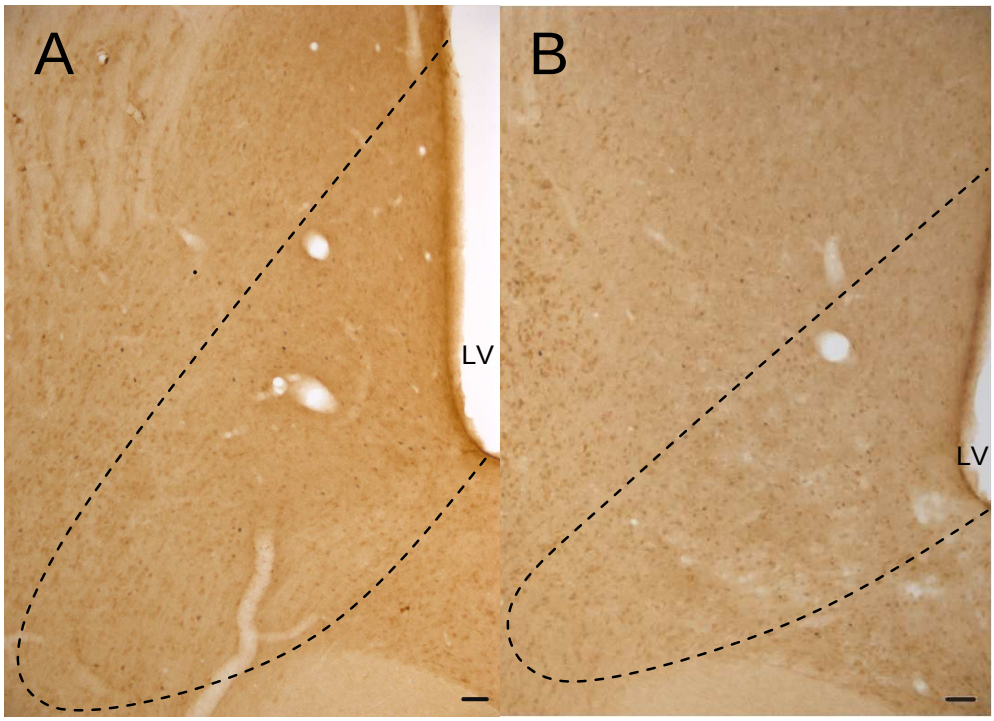


Figure 4.10: Photographs of activated oxytocin receptor containing cells in the bed nucleus of the stria terminalis of lactating rats after a maternal aggression test. Double immunocytochemistry was performed for Fos and oxytocin receptor (OTR) in the brain of a lactating rat following either a 30 min maternal aggression test or left undisturbed in their home cage with pups present. Photographs depict Fos positive OTR containing cells in the bed nucleus of the stria terminalis (defined by black dashed lines) in the non aggression and aggression tested (A and B respectively) lactating rat. High power photographs show Fos and OTR positive cells (black arrows) in non aggression and aggression tested lactating rats (C and D respectively) Scale bars = 50µm. Abbreviation: LV = lateral ventricle



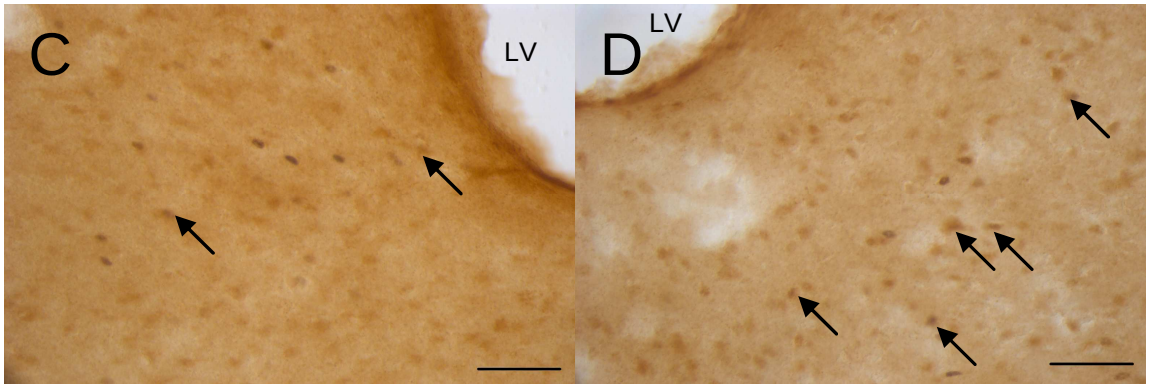


Figure 4.11: Photographs of activated oxytocin receptor containing cells in the lateral septum of lactating rats after a maternal aggression test. Double immunocytochemistry was performed for Fos and oxytocin receptor (OTR) in the brain of a lactating rat following either a 30 min maternal aggression test or left undisturbed in their home cage with pups present. Photographs depict Fos positive OTR containing cells in the lateral septum (defined by black dashed line) in the non aggression and aggression tested (A and B respectively) lactating rat. High power photographs show Fos and OTR positive cells (black arrows) in non aggression and aggression tested lactating rats (C and D respectively). Scale bars = 50µm. Abbreviation: LV = lateral ventricle.

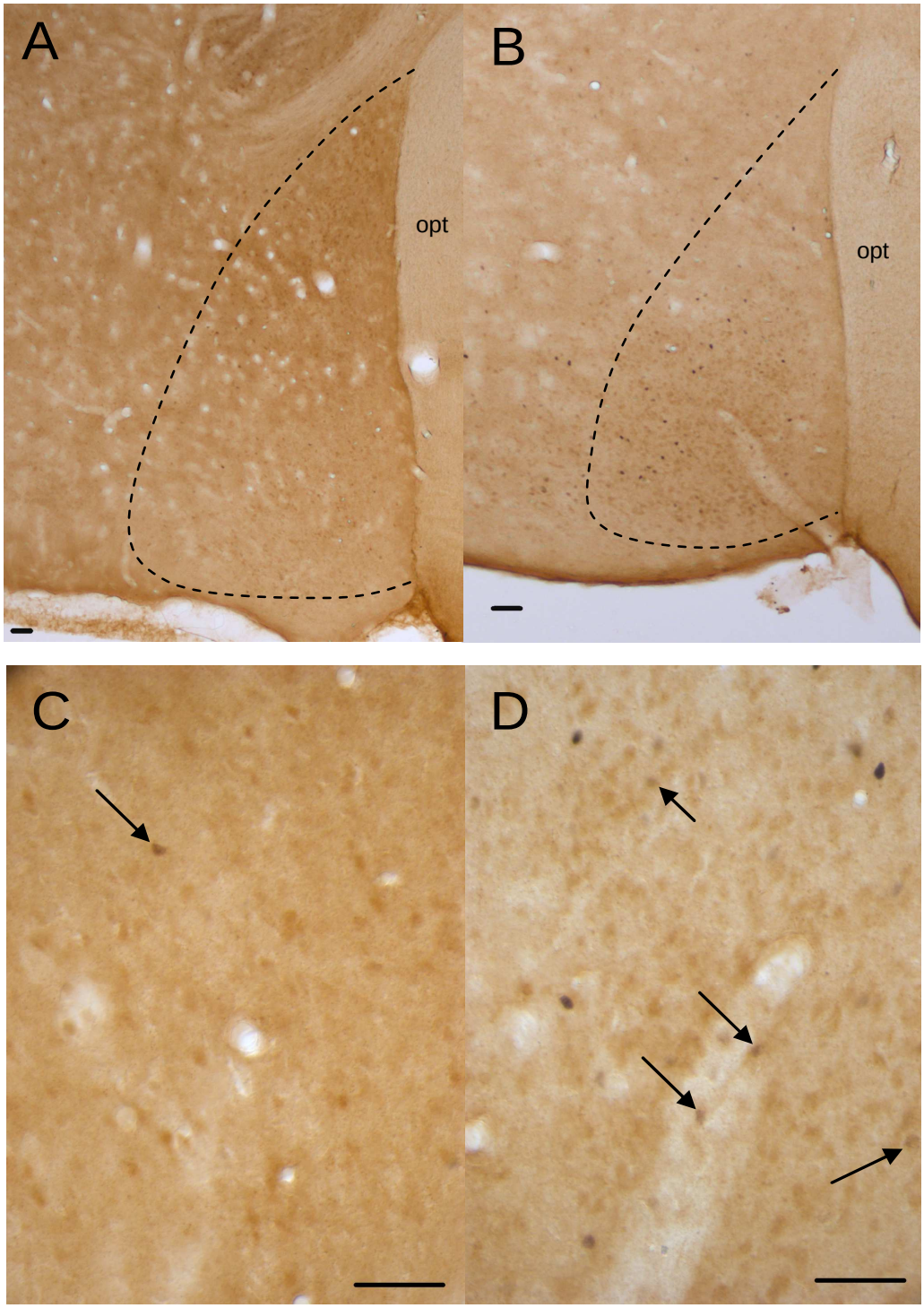


Figure 4.12: Photographs of activated oxytocin receptor containing cells in the medial amygdala of lactating rats after a maternal aggression test. Double immunocytochemistry was performed for Fos and oxytocin receptor (OTR) in the brain of a lactating rat following either a 30 min maternal aggression test or left undisturbed in their home cage with pups present. Photographs depict Fos positive OTR containing cells in the medial amygdala (defined by black dashed line) in the non aggression and aggression tested (A and B respectively) lactating rat. High power photographs show Fos and OTR positive cells (black arrows) in non aggression and aggression tested lactating rats (C and D respectively) Scale bars = 50µm. Abbreviations opt=optic tract.

4.6 Vasopressin binding and vasopressin V1a mRNA receptor expression throughout the peri-partum period: comparison to aggressive behaviour

4.6.1 Method

Brains which had been collected from rats in the first experiment of chapter 4 were serially sectioned using a cryostat (for details see chapter 2). Sections were then processed for either AVP-R autoradiography (chapter 2) in the LS, BnST, MPOA, PVN, MeA and CeA or AVP V1a receptor in situ hybridisation (chapter 2) in the MeA, CeA and PVN. Films for AVP-R autoradiography were left to expose in the dark room for 6 days before being developed. Slides dipped in emulsion for AVP V1a receptor ISH were left to expose for 10 weeks before being developed with test slides having been developed at 8, 9 and 10 weeks.

4.6.2 Results

4.6.2.1 Vasopressin receptor binding

There was significantly lower AVP-R binding in the MeA for the PD21 group compared to the LD14 group ($p=0.003$, $H_4=15.7$); no statistical differences were observed between the PD16/19/20, PD or LD4-7 groups ($p>0.05$, $H_4=15.7$; Fig. 4.13). For analysis of AVP-R as described in Chapter 2 Section 2.8.3, for each rat an average was taken from analysing at least four sections. Unfortunately for the LD21 group, there were not enough sections per rat to be able to perform statistical analysis. Conversely to the MeA, in the CeA AVP-R binding was significantly higher in the PD group compared to LD21 ($p=0.037$, $H_5=11.8$); no other group was statistically different ($p>0.05$, $H_5=11.8$; Fig. 4.13). Within the PVN, the PD group had significantly greater AVP-R binding compared to LD4-7 ($p=0.014$, $H_5=14.2$) but no other group ($p>0.05$, $H_5=14.2$; Fig. 4.13). Unlike the OTR binding, there was no

significant difference in AVP-R binding within the LS ($p=0.052$, $H_5=11.0$), BnST ($p=0.98$, $H_5=0.68$) or MPOA ($p=0.28$, $H_5=6.2$) between any groups (Fig. 4.13).

4.6.2.2 Vasopressin V1a mRNA receptor expression

AVP V1a receptor mRNA expression was examined in the MeA, CeA and PVN regions of the brain. Within the CeA, significantly higher levels of V1a mRNA expression were observed in the PD group compared to the PD16/19/20 ($p=0.005$, $H_5=16.8$) and PD21 ($p=0.005$, $H_5=16.8$) groups but not with any the lactation day groups ($p>0.05$, $H_5=16.8$; Fig. 4.14). No significant differences were observed within the MeA ($p=0.076$, $F_{(5,46)}=2.16$), magnocellular ($p=0.46$, $F_{(5,29)}=0.96$) or parvocellular ($p=0.36$, $F_{(5,29)}=1.14$) PVN between any of the groups (Fig. 4.14).

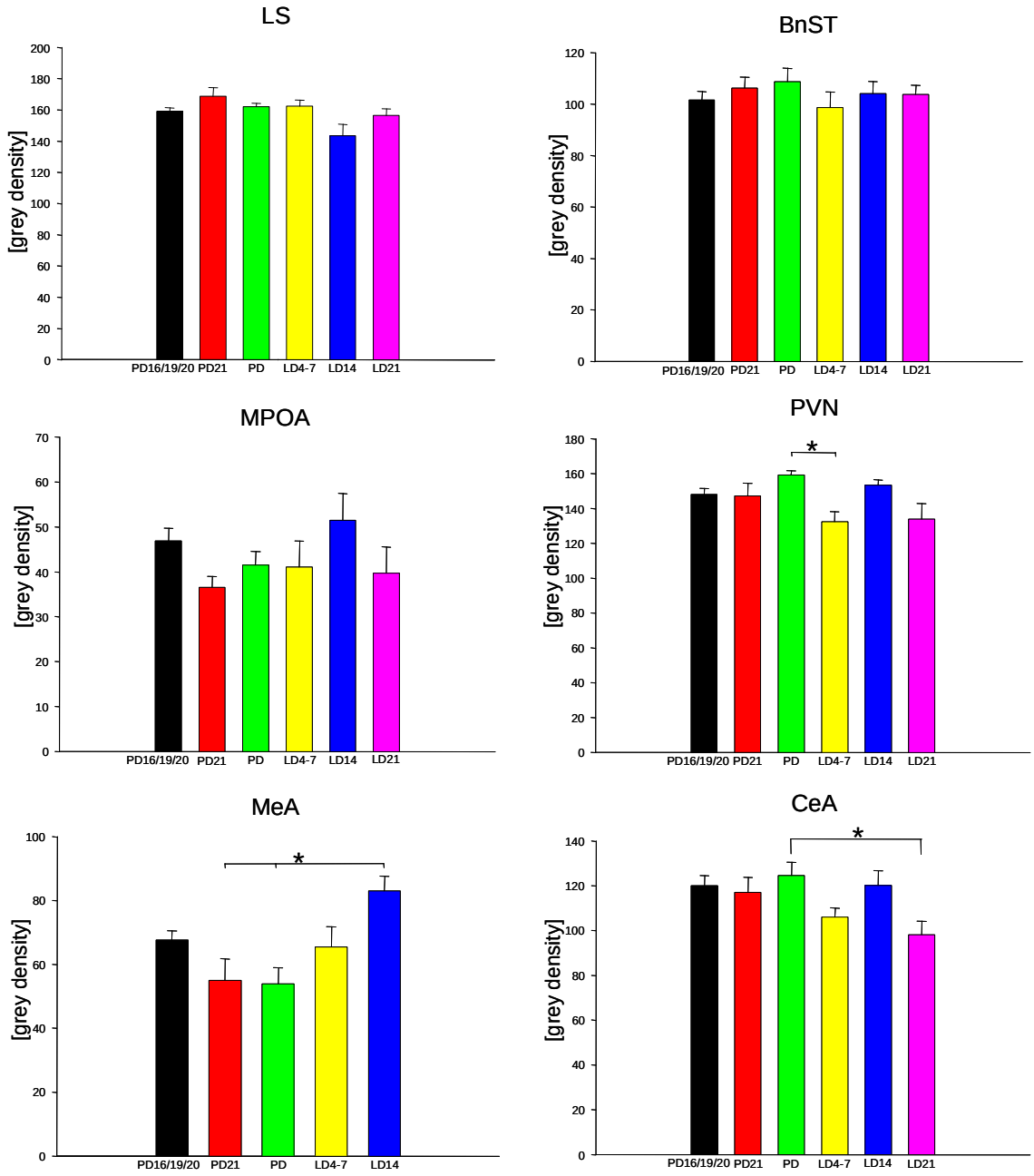


Figure 4.13: Average vasopressin receptor binding in specific brain regions of rats through pregnancy, parturition and lactation following a maternal aggression test. All rats performed a 10 min maternal aggression test followed by immediate decapitation. Brains were collected and frozen on dry ice before being processed for vasopressin receptor (AVP-R) binding using receptor autoradiography. AVP-R binding was examined in the lateral septum (LS), bed nucleus of stria terminalis (BnST), medial preoptic area (MPOA), supraoptic nucleus (SON), medial and central amygdala (MeA and CeA respectively) of rats tested on (PD16/19/20, n=21) or 21 (PD21 n=7), day of parturition (PD, 1h after birth of last pup; n=7) or lactation day 4-7 (LD4-7, n=6), 14 (LD14, n=7) and 21(LD21, n=8). Data are represented as mean +SEM. *=p<0.05

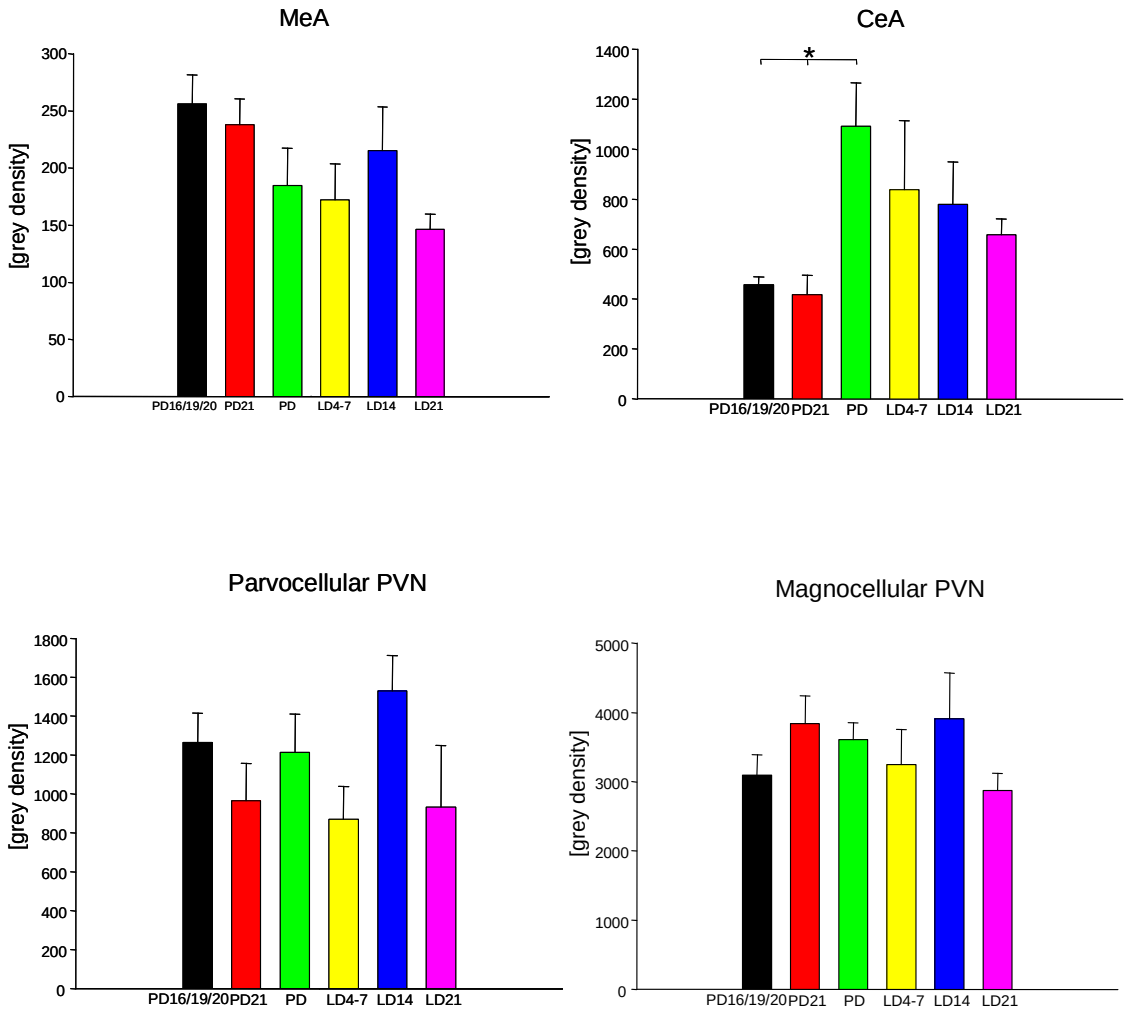


Figure 4.14: Average vasopressin V1a receptor mRNA expression in specific brain regions of rats through pregnancy, parturition and lactation following a maternal aggression test. All rats performed a 10 min maternal aggression test followed by immediate decapitation. Brains were collected and frozen on dry ice before being processed for vasopressin (AVP) V1a mRNA expression using insitu hybridisation. AVP V1a receptor mRNA expression was examined in the paraventricular nucleus (PVN; parvocellular and magnocellular), medial and central amygdala (MeA and CeA respectively) of rats tested on pregnancy day 16, 19, 20 (PD16/19/20, n=21) or 21 (PD21 n=7), day of parturition (PD, 2h after birth of last pup; n=7) or lactation day 4-7 (LD4-7, n=6), 14 (LD14, n=7) and 21(LD21, n=8). Data are represented as mean +SEM. *=p<0.05. Analysis of the amygdala was done by student, Luke Birch.

4.7 Correlation of maternal aggression with central vasopressin secretion

4.7.1 Method

The method was the same as above for the OXT microdialysis experiment. Dialysates for examination of AVP secretion were collected from the LS (PD19, n=8; PD21 n=8), BnST (PD19, n=8; PD21 n=8), PVN (PD19, n=8; PD21, n=8) and MeA (LD4, n=7) of rats that experienced a 10 min maternal aggression test.

4.7.1.1 Statistics

A two-way ANOVA was performed to determine if there was a statistical difference between samples within a group or across groups. If data was significant ($p<0.05$), a Tukey post hoc analysis test was done.

4.7.2 Results

4.7.2.1 Vasopressin secretion

AVP secretion in the PVN was observed in the PD19 group during a maternal aggression test ($p=0.009$, $F_{(4, 70)}=6.19$ for sample 3 vs sample 2) but not in the PD21 group ($p>0.05$, $F_{(4, 70)}=6.19$; Fig. 4.16). There was no significant difference between samples across groups for the release of AVP within the PVN ($p=0.63$, $F_{(1, 70)}=0.24$). Within the LS, there was also no significant difference across samples between the PD19 and PD21 groups ($p=0.089$, $F_{(1,70)}=2.97$). However for both groups, comparisons across samples within group showed sample 5 was significantly higher (PD19, $p=0.019$ vs sample number 2; PD21 $p=0.031$ vs sample number 4; Fig 4.15).

No significant change was observed in the release of AVP in the BnST across samples within the PD19 ($p=0.844$, $F_{(4,65)}=0.35$) or the PD21 ($p=0.844$, $F_{(4,65)}=0.35$) groups (Fig. 4.15). However, there was a significant difference in the release of AVP in the BnST between tbe PD19 and PD21 groups for sample number 4 ($p=0.046$) and

5 ($p=0.038$) (Fig. 4.15). The MeA was examined for the LD4-7 group and no detectable significant change in release of AVP was observed during a maternal aggression test ($p>0.05$, $x^2=12.43$; Fig. 4.16).

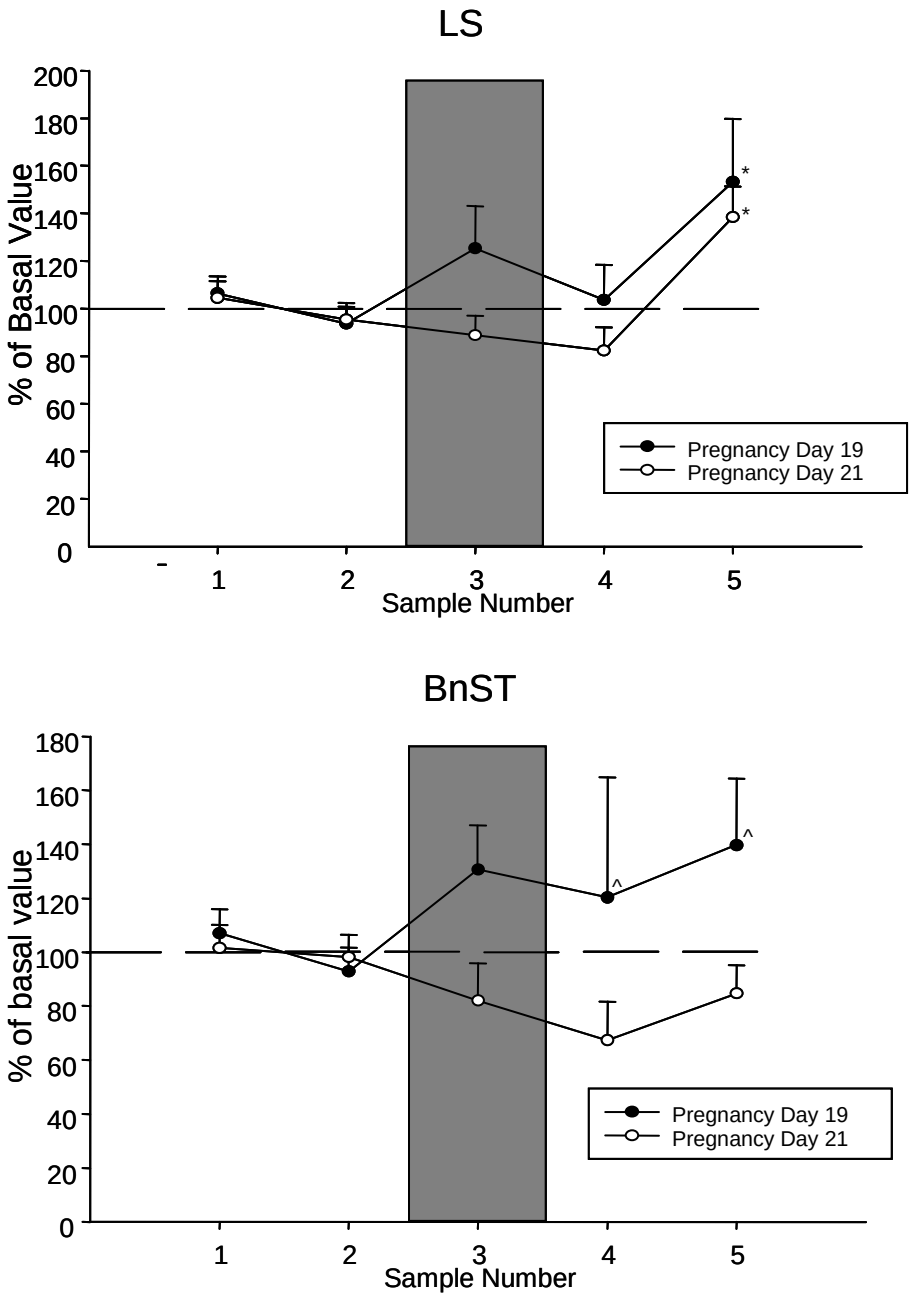


Figure 4.15: Vasopressin release within the lateral septum and bed nucleus of stria terminalis of the resident rat during a maternal aggression test. Bilateral microdialysis surgery was performed two days prior to maternal aggression testing on all pregnant rats. On the day of testing (pregnancy day 19 [PD19] or 21 [PD21]), rats were connected to microdialysis pumps and left to acclimatize for 2h, five dialysates were then collected every half an hour. The first two were collected whilst the rat was left undisturbed in her home cage. The third sample was collected during 10 min maternal aggression test. The final two samples were collected with the rat left undisturbed in her home cage. All dialysates once collected were immediately frozen on dry ice until quantification for vasopressin by radioimmunoassay. Microdialysis probes were implanted into the lateral septum (LS; PD19 n=8, PD21 n=8) or bed nucleus of stria terminalis (BnST; PD19 n=7, PD21 n=8). ^=$p<0.05$ when comparing sample across groups. *=$p \leq 0.05$ when samples within the group. Data are represented mean + SEM.

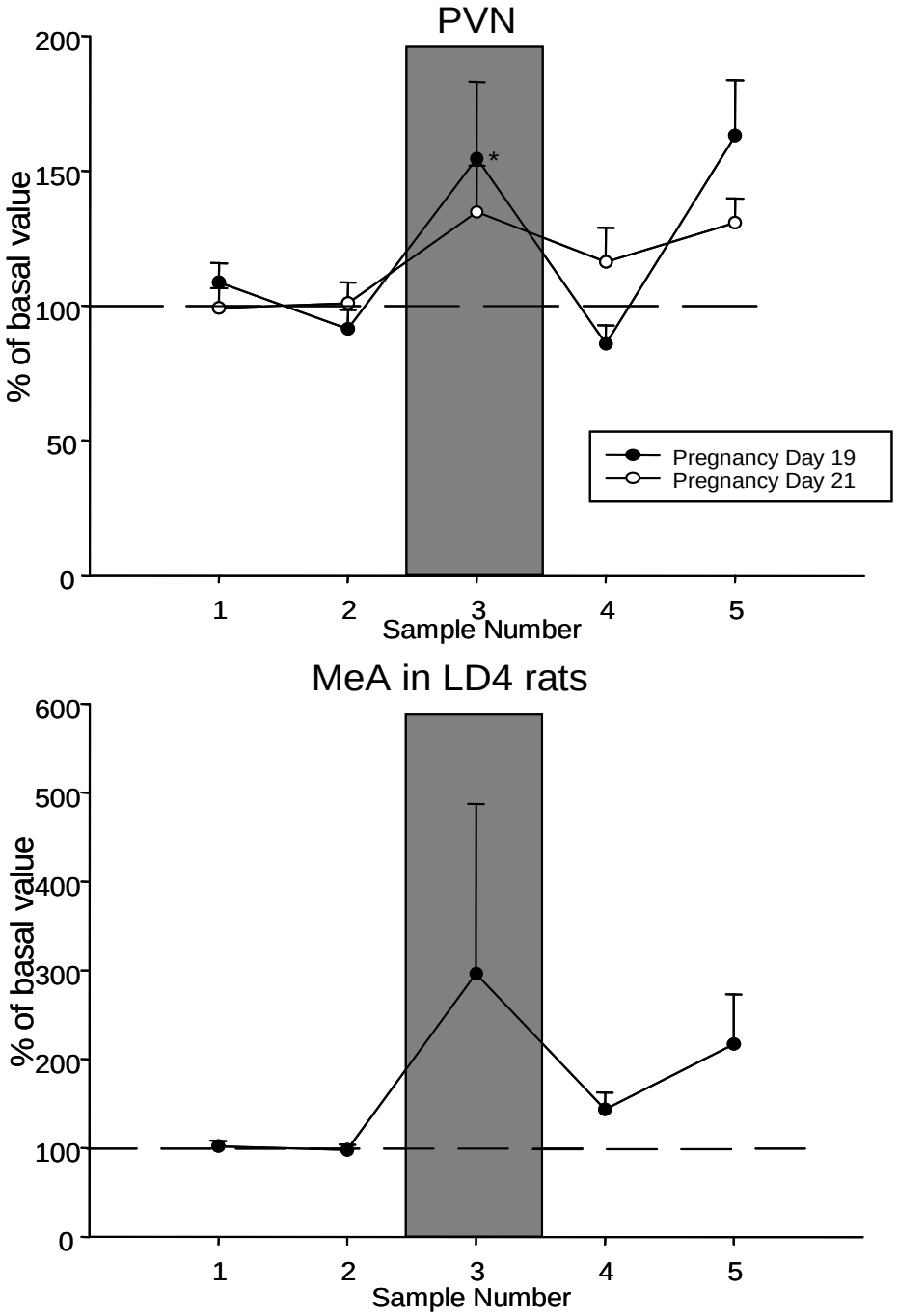


Figure 4.16: Vasopressin release within the paraventricular nucleus and medial amygdala of the resident rat during a maternal aggression test. Bilateral microdialysis surgery was performed two days prior to maternal aggression testing on all pregnant and lactating rats. On the day of testing (pregnancy day 19 [PD19] or 21 [PD21] or lactation day 4 [LD4]), rats were connected to microdialysis pumps and left to acclimatize for 2h, five dialysates were then collected every half an hour. The first two were collected whilst the rat was left undisturbed in her home cage. The third sample was collected during 10 min maternal aggression test. The final two samples were collected with the rat left undisturbed in her home cage. All dialysates once collected were immediately frozen on dry ice until quantification for vasopressin by radioimmunoassay. Microdialysis probes were implanted into the paraventricular nucleus (PVN; PD19, n=8; PD21, n=7) or medial amygdala (MeA; LD4, n=7). *=p≤0.05 when sample 3 compared to sample 2. Data are represented mean + SEM.

4.8 Discussion

In this chapter, a clear and detailed picture of the changes in maternal aggression throughout the peri-partum period has been built. Different levels of aggressive behaviours are expressed throughout the peri-partum period however full maternal aggression involving fierce attacks and all aggressive behaviour is only first displayed on PD21. A study by Mayer and Rosenblatt (1984, [180]) did not observe an increase in nest defence in rats until PD22, just hours prior to parturition. As the rats in our study were killed immediately after the maternal aggression test, it is unknown how close to term they were. However it would be fair to assume they were within 24h or so therefore within the time period that Mayer and Rosenblatt mention for increased nest defence [180]. Thus these two results do concur that just prior to birth maternal aggression is expressed for the first time.

Maternal aggression then dips slightly just after parturition where it appears more beneficial for pup survival to elicit nurturing rather than attacking behaviour. Flannelly *et al* (1986, [168]) observed a higher level of maternal aggression on the day of birth compared to the results of study but this may depend on the time point of testing; rats in this study were almost immediately after birth (1h after last pup birth) whilst in the Flannelly study testing was done several hours after birth. One factor common to both studies is the very short latency to attack on the day of parturition. Maternal aggression peaks during the first week of lactation as observed in previous studies before gradually diminishing to disappear on LD21 [168, 179]. This constructed model (Fig. 4.17) can now be used in correlating hormonal changes of the peri-partum period with variations in maternal aggression expression for rats.

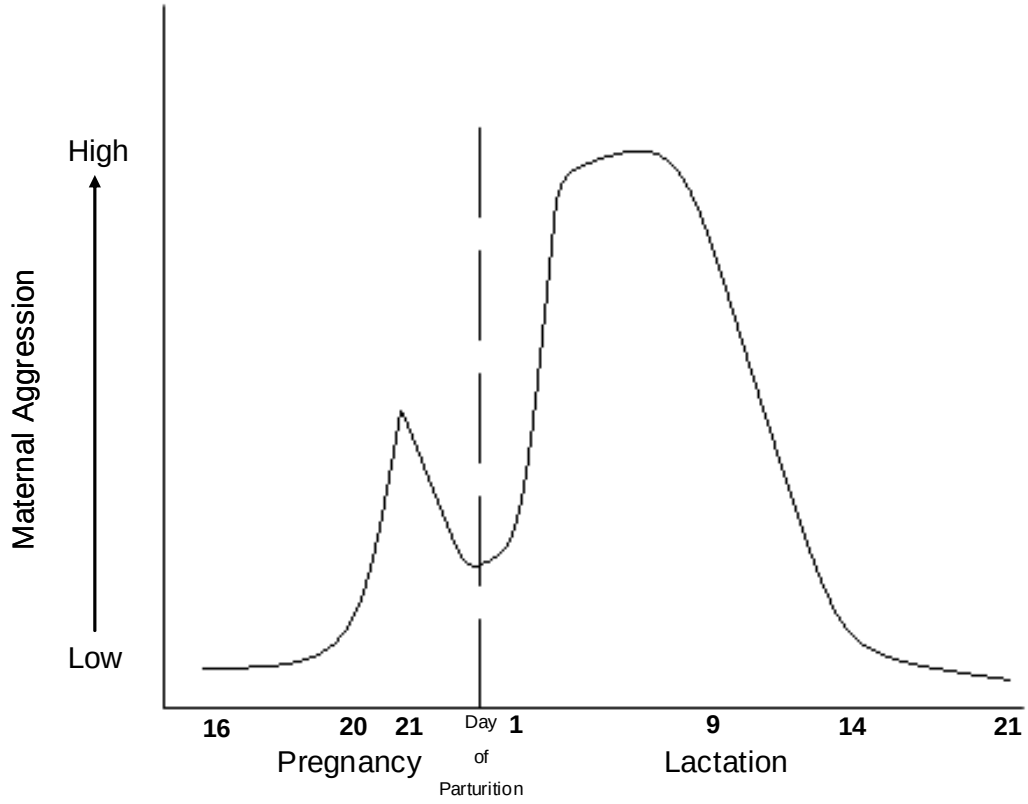


Figure 4.17: The display of maternal aggression through the peri-partum period in the rat. In the rat, full display of maternal aggression first appears in late pregnancy (pregnancy day 21). Maternal aggression then diminishes slight on the day of parturition before peaking during the first week of lactation. It then gradually reduces until it disappears around weaning (lactation day 21).

In this study, this model was used to investigate both the OXT and AVP systems during the peri-partum period. Dynamic changes were observed in both relating to maternal aggression providing evidence of their important but very different roles in the control of maternal aggression and behaviour.

In the OXT system, OTR binding was found to be significantly higher in the BnST on PD21 when maternal aggression is first expressed in full. The BnST is thought to be important in the long term control of anxiety via both indirect projections through the PVN and amygdala and direct projections to the PAG [14, 105]. In addition, Meddle *et al* (2007, [307]) observed OTR mRNA expression and

activation of OTR expressing cells in the BnST to increase just after parturition (90 min after the birth of the first pup). These findings plus the observed higher levels of OTR binding on PD21 in the current study, the day before expected parturition, suggest that OXT may reduce anxiety and fear in preparation for the pup arrival so the mother will approach her pups immediately after birth enabling the formation of a bond and attachment to occur between mother and offspring.

The changes in OTR binding within the BnST supports the two theories about OXT actions around parturition; the first that OXT is necessary for onset of maternal behaviour but not maintenance [316]. The higher levels of OTR binding on PD21 return to normal by LD4-7. This theory is further supported by higher levels of OTR binding on PD in the MPOA in our study which decreased by LD4-7 to levels comparable to pregnancy. In addition, it has been observed that OTR mRNA expression increased and more OTR expressing neurons are activated within the MPOA just following parturition [307]. The MPOA is an area important in maternal behaviours as electrolytic lesions here disrupt all forms of maternal care [80, 82, 438].

The second theory is that OXT by modulating fear and anxiety indirectly helps maintain maternal aggression [265]. During pregnancy, OXT secretion was increased within the PVN during a maternal aggression test therefore one could hypothesise that OXT works to reduce anxiety to ensure aggressive behaviour from the beginning of maternal aggression expression (PD21). Furthermore, activation of OTR containing cells was observed to be increased within the BnST after a maternal aggression test on LD4-7; the time point when maternal aggression is highest. This suggests that the actions of OXT on maternal aggression regulation within the BnST

are there from the start of maternal aggression and still apparent at the time of maternal aggression peak.

The LS is an area which appears to be important in the maternal aggression circuitry but not necessarily essential for the expression of other maternal behaviours [12, 194, 196]. Results from this study would further highlight its importance in the maternal aggression circuitry and suggest that it is highly influence by the OXT system. An increase in OTR binding is observed within the LS on at the peak of maternal aggression (LD4-7). Additionally, an increase in the activation of OXT sensitive cells in the LS in aggressive lactating rats compared to non aggressive was observed providing further evidence that OXT is involved in the regulation of maternal aggression at the time of its greatest expression within the LS. However, no difference was observed in OXT secretion on PD19 and PD21 during a maternal aggression test within the LS and previous research has observed no significant change in OXT secretion in lactating rats (at the peak of maternal aggression peak) compared to virgin female rats during an maternal aggression test [424]. Thus it would appear in the LS, it not a change in the release of OXT regulating maternal aggression but activation of its receptors. Examination of OTR mRNA expression in the LS throughout the peri-partum period would help to substantiate this idea. As no change was observed in LS in AVP-R binding through out the peri-partum period or AVP secretion during a maternal aggression test, control of maternal aggression within the LS would appear to be under OXT influence. Future research examining the effects of direct application of selective OXT antagonists and antisense oligonucleotides, which specifically target OXT or OTR to decrease their expression,

to the LS would help to further elucidate more clearly the role of OXT in maternal aggression within the LS [200].

Another area highlighted by Fos studies as important in the maternal aggression circuitry is the PVN [188]. The PVN is a crucial component in the stress axis response and has already been shown to be an area where OXT and AVP work against each other to create a balance in response to stress [335]. It is established that OXT secretion increases in the PVN during a maternal aggression test at LD4 when maternal aggression is at its highest and manipulations of OXT within the PVN can affect maternal aggression expression [320, 439]. This study provides further evidence that OXT is the important peptide controlling anxiety and fear around the peri-partum period within the PVN. No change in AVP V1a receptor mRNA expression in either magnocellular or parvocellular PVN throughout the peri-partum period were observed whereas within the magnocellular PVN activation of OTR containing cells was seen to increase immediately postpartum [307]. Furthermore, a decrease in the AVP receptor binding in the PVN on LD4-7 would suggest that OXT works to decrease the stress axis response to enable maternal aggression. Examination of OTR binding within the PVN during the peri-partum period would provide further evidence of the importance of OXT in maternal aggression regulation in the PVN. However an increase in the release of AVP within the PVN was seen on PD19 but not PD21 when maternal aggression is first expressed. This is in contrast to OXT where significant increases in release are observed on PD19, PD21 and during the first week of lactation [439]. Studies to determine whether selective AVP antagonists or antisense AVP oligonucleotides applied directly to the PVN affect

maternal aggression would be able to provide further evidence of whether OXT is the crucial peptide working control maternal aggression within the PVN.

Interestingly within the CeA, an area important in the control of fear and anxiety, not only was there an increase in AVP-R binding on PD but also an increase in AVP V1a mRNA expression [265, 266]. These changes, along with no change in CeA OTR binding, could lead to the proposal that in the CeA, the influence of AVP is more important in the control of maternal aggression and maternal behaviour around the peri-partum period. However previous studies have clearly defined OXT effects on maternal aggression within the CeA in the lactating rat; with direct OXT application decreasing maternal aggression and OXT antagonist increasing [431, 432]. Also, OXT release within the CeA is increased during a maternal aggression test [152]. Within the CeA, distinct areas are under OXT or AVP control and it has been demonstrated that the area under OXT control projects via GABAergic neurons to the AVP inducing fear neurons thereby reducing fear expression [266, 440].

It is interesting therefore that AVP V1a mRNA receptor expression and AVP-R binding both increased on PD which would lead to the assumption fear expression is increased at this time. As binding decreases to pre-parturition levels by LD4-7 one could hypothesise that the increase AVP binding in the CeA causes an increase in fear in response to an intruder specifically after parturition to help with creating a strong mother and pup bond. Lactating rats on PD spend more of their time with engaging with their litter during a maternal aggression test than displaying aggressive behaviour. Hence further research is required to elucidate the role of OXT and AVP in the CeA and how the two hormones work to balance the effects of each other. Interestingly, as discussed in chapter 4 and 7, a neurosteroid, AP, which had been

implicated in regulation fear, appears to increase activation of the CeA during maternal aggression [105]. Further evidence that control of the CeA in regulating fear is important in enabling the display of maternal aggression and possibly the actions of OXT and AP either work together or provide back-up in case one fails in its actions.

In the MeA, OXT is proposed to important for the attainment of social memories and hence essential for social recognition as OXT KO male mice fail to recognise the same female during the second social test unless pretreated with an OXT injection into the MeA [425]. Furthermore, in these socially tested OXT KO mice activation of the main and accessory OBs was the same as normal mice but activation was decreased in the MeA, BnST and MPOA [425]. The MeA receives information from the OBs and then passes it onto the BnST and MPOA so these results suggests information is relayed as normal until the MeA where it is disrupted [425]. It could be proposed therefore that the increase in activation of OTR expressing cells in the MeA during a maternal aggression test reflects the processing of chemosensory information from the pups and intruder so the intruder is recognised as novel and a potential threat to the pups. OTR mRNA expression is observed to significantly increase just after parturition possibly reflecting the initiation of the OXT system so social memory of the litter is obtained [307].

In non-maternal virgin rats, the MeA is proposed to work to inhibit maternal behaviour [70]. Examination of Fos in non maternal but pup exposed virgin rats compared to fully maternal pup-sensitized rats showed activation was increased in anterior hypothalamic nucleus (AHN), principal BnST (BnSTpr), ventral LS (LSv), dorsal premammillary nucleus (PMd) and dorsomedial and central areas of the

ventromedial hypothalamic nucleus (VMNdm,c) [70, 98]. These areas all receive inputs from the MeA thus it was proposed that the MeA inhibits maternal behaviour in virgin rats [70]. Lesions of the MeA resulted in reduced Fos activation in AHN, VMN, BnSTpr, LSv and PMd in non-maternal virgin rats and lesions in the MeA along with AHN and VMN lesions stimulated maternal behaviour further evidence of that the MeA through the AHN and VMN (and possibly the BnSTpr, LSv and PMd) works to inhibit maternal behaviour [40]. Our study showed increased OTR cell activation in the MeA after a maternal aggression test which could lead to the suggestion that OXT during the lactation period works to disinhibit the MeA to allow the display of maternal behaviours including maternal aggression. Research investigating the effects of direct MeA OXT antagonist application to lactating rats or OXT to non-maternal virgin rats would test this theory. One would hypothesise that an OXT antagonist would allow the MeA to actively inhibit maternal behaviour in lactating rats whereas OXT in non-maternal virgin rats would promote maternal behaviour.

It is important to remember that when interpreting changes in AVP or OXT release that it is not known where or on which receptors they are acting on. The same is true for activation of OTR containing cells, it is unknown which peptide may be activating these cells. This is because the AVP and OXT peptides have similar structures and are therefore able to act on each other receptors resulting in cross interactions [200]. Thus current and future research involving antisense oligonucleotides specific to each peptide and their receptors, gene transfer virals (which cause overexpression of AVP or OXT receptors), KO and transgenic animals will help to piece together the puzzle over the specific actions of AVP and OXT

during the peri-partum period [200]. The generation of OXT KO mice have already been observed to express normal maternal behaviour hence more evidence that OXT is not necessary for maternal behaviour maintenance [316]. Antisense oligonucleotides have already been proven to cause brain region specific changes resulting in different behavioural outcomes, application of these oligonucleotides in brain regions important for maternal aggression (e.g. OXT in the LS) or maternal behaviour (e.g. AVP in the MPOA) would help elucidate further the precise roles of OXT and AVP in maternal behaviour [200].

In this study, we have observed that there are specific changes in OXT and AVP systems in connection to the expression of maternal behaviour including aggression. OTR binding is increased in the BnST and MPOA at parturition whereas AVP V1a receptor binding is significantly higher in the CeA possibly indicating where each neuropeptide may work to induce the rapid onset of maternal behaviour immediately after parturition. During maternal aggression expression, OXT release is significantly increased in the BnST, PVN and SON with higher numbers of OTR containing cells activated in the LS, BnST and MeA following a maternal aggression test also observed. AVP secretion was increased in PVN during maternal aggression testing, but at the peak of maternal aggression (LD4-7) a decrease in AVP V1a receptor binding was observed. These results indicate that these two neuropeptides work together in influencing distinct brain regions to enable the correct expression of maternal behaviour.

Chapter Five: Maternal behaviour and the neurosteroid, allopregnanolone.

5.1 Introduction

Neurosteroids were discovered during the past decade when the brain was found to be able to synthesise peripheral steroids *de novo* [360, 362, 441, 442]. These steroids can regulate the main excitatory, glutamate, and inhibitory, γ-aminobutyric acid (GABA), neurotransmitters and their receptors of the central nervous system so they have a direct influence on a broad range of behaviours from anxiety to cognition and hence why they became known as neuroactive steroids or neurosteroids [358, 359, 361, 441]. The progesterone metabolites, specifically 5α-dihydroprogesterone (5α-DHP) and 3α, 5α-tetrahydroprogesterone (3α, 5α-THP or allopregnanolone, AP), have been found to be potent positive modulators of the $GABA_A$ receptors [356-358]. By enhancing the effect of the $GABA_A$ receptors functioning, they impact upon GABA's broad range of behavioural effects from being anticonvulsant, anxiolytic to anaesthetic [358]. The sulphated versions of neurosteroids, for example dehydroepiandrosterone sulphate (DHEAS), however exhibit a negative modulatory control on $GABA_A$ receptor functioning [342]. Thus, there is a balance between the two types of neurosteroids as one enhances the effects of GABA causing a decrease in anxiety and stress behaviour, the other dampens GABAs effect resulting in the opposite behavioural outcomes.

The impact of neurosteroids on $GABA_A$ receptors is influenced by local steroid synthesis and metabolism, subunit composition of the receptor and phosphorylation mechanisms [356]. Although the subunit composition is known to change through pregnancy, parturition and the onset of lactation, this effect on the functioning of the receptor and how it changes the influence of neurosteroids upon behaviour is as yet

unclear [346, 443]. Many researchers have put forward an argument that regardless of the subunit composition, the most important influence on $GABA_A$ receptor functioning is its phosphorylation state [444]. Koksma *et al* (2003) found an increased level of phosphorylation creates insensitivity of $GABA_A$ receptors to 5α-DHP specifically within the SON [444]. It was also found this $GABA_A$ receptor insensitivity occurred around the same time (i.e. at parturition, lactation has not been investigated yet) as the increase in OXT release therefore it seems reasonable to propose that OXT may play a role in creating $GABA_A$ receptor insensitivity to AP.

Previous research has found the modulation of $GABA_A$ receptor functioning by neurosteroids can influence physiology and behaviour in many ways. These can range from the blood volume control during pregnancy, stress responses, seizure susceptibility to anxiety and depression [105, 199, 366, 368-370, 372, 441, 445-448]. Anxiety related disorders, for example, are thought to partly result from a dysfunction in the central GABAergic system [449]. Many anxiolytic drugs act by enhancing $GABA_A$ receptors actions, by a similar mechanism to neurosteroids. AP has been found to mediate the actions of one such drug, etifoxine, on anxiety behaviour in rodents [449]. AP is not only implicated in having a role in anxiety but also other related disorders such as premenstrual tension, post-traumatic stress and other social behaviour during periods of hormonal upheaval [358, 361, 381, 449]. AP has also been found to attenuate the stress response through influencing the actions of $GABA_A$ receptors on the OXT secretion in males and in females during late pregnancy [199, 446]. Interestingly one type of seizure, known as catamenial epilepsy, occurs at specific times during the menstrual cycle when there is a decrease in circulating progesterone levels [366]. Moreover, AP levels can be inversely correlated with

seizure frequency in these catamenial epileptic women [366]. Thus, there are many roles for AP actions on $GABA_A$ receptor functioning in the body across the menstrual cycle and during the peri-partum period when huge changes in progesterone occur. Despite much research focusing on elucidating these functions, few investigations have examined the impact of neurosteroids on maternal behaviour.

The disinhibition of $GABA_A$ receptors just prior to parturition allows OXT secretion to increase necessary for normal parturition and lactation; this occurs at the same time as the start of maternal behaviour [352, 431]. Although $GABA_A$ receptors have an effect upon many different behaviours from anxiety to cognition, the influence of $GABA_A$ receptors during the peri-partum period upon maternal behaviour is presently undefined but there is evidence for a regulatory role of GABA. In a study by Lee and Gammie (2007, [158]), they demonstrated in lactating mice that $GABA_A$ receptor agonists enhance maternal aggression. However the literature is conflicting as direct infusions of $GABA_A$ receptor agonists into the MPOA significantly reduced aggression [158, 438]. This disparity may be due to species differences as mice were used in the former and rats in the latter study. Differences between mice and rats have been observed in innate anxiety where rats with high anxiety are more aggressive and for mice the converse is true [204, 205, 320]. The impact of $GABA_A$ receptor agonists on maternal aggression could also be brain site specific as maternal aggression is enhanced when $GABA_A$ receptor agonists are injected into LS but aggression is decreased following injection into the MPOA and BnST [158]. Thus, much research is required to clearly identify the role of GABA in maternal aggression. One area that is yet to be explored is the modulation of $GABA_A$ receptors by neurosteroids, especially by AP.

In male rodents, aggressive behaviour has been clearly linked to increased levels of AP [342, 373, 378-380]. AP was investigated because there was irrefutable data that GABAergic transmission is essentially involved in the regulation of aggression and AP is the most potent positive modulator of $GABA_A$ receptors known [379, 450]. Also, down regulation of endogenous AP synthesis was linked with reduced $GABA_A$ receptor signal transduction [450]. AP was discovered to have a bi-tonic effect on aggression with low doses having no effect, and high doses being sedative, whereas moderate doses significantly increased the aggressive behaviour in male mice [342]. Thus, there is strong evidence for AP role in the control of aggressive behaviour in the brain of males however this is yet to be investigated in females.

The aim of the experiments in this chapter is therefore to investigate how neurosteroids, specifically AP, impact upon maternal behaviour. The dramatic fall in progesterone levels towards the end of pregnancy occurs at the same time as the rapid increase in synthesis and release of OXT necessary for parturition [307, 353, 451, 452]. At this time, maternal behaviour begins to be expressed [1, 3, 51]. Hence, it is proposed that progesterone withdrawal allows the timed release of OXT which then goes on to drive maternal behaviour. For example, if progesterone levels are maintained to the end of pregnancy, post partum females do not express maternal behaviour [353]. Also, if progesterone is injected into estrogen primed, ovariectomised virgin females the onset of maternal behaviour is delayed or even abolished [353]. The mechanism by which progesterone exerts its effect still requires elucidation.

This current study tests the hypothesis that that progesterone drives maternal behaviour via the actions of its neurosteroid metabolite, specifically AP, on the $GABA_A$ receptors. This hypothesis is built on a number of reasons. (1) It is known that AP mimics the levels of progesterone during pregnancy and parturition [453]. (2) The SON is one area highly associated with maternal behaviour functioning and has a large number of $GABA_A$ receptors with inhibitory control over release of OXT before parturition to which AP is known to contribute [320, 454]. (3) OXT can cause insensitivity $GABA_A$ receptors to 5α-DHP thus allowing for the withdrawal of GABA inhibition coinciding with the expression of maternal behaviours during late pregnancy [444]. (4) Around parturition, the same time as the significant increase in OXT levels, there are dramatic changes in OXT receptor expression and functioning especially in areas, specifically the MPOA, BnST and SON, related to maternal behaviour and which also have a clear GABA functioning component [307].

5.2 The effects of allopregnanolone on maternal behaviour during lactation

The first aim of this study was to test the hypothesis that the neuroactive steroid AP will inhibit the expression of maternal behaviour including the display of maternal aggression.

5.2.1 Method

Lactating rats were randomly assigned into one of four groups; AP injected and maternal aggression tested (APA), AP injected and maternal behaviour observed (APNA); vehicle injected and maternal aggression tested (VA) or vehicle injected and maternal behaviour observed (VNA). APA (n = 9) and APNA (n = 9) rats were injected s.c. 3mg/kg AP (Steraloids Inc.Ltd London; 6 mg AP in 12% ethanol and

corn oil) at least 20h prior and 1mg/kg AP (2 mg AP in 12% ethanol and corn oil) 2h prior to being exposed to a 30 min maternal aggression test or maternal behaviour observation (as described in Chapter 2). This dosage was chosen because it has already been shown to have significant actions on the stress response within pregnant rats [199, 352]. Also, by injecting 20h and 2h before behavioural testing allowing for both long term genetic changes and short term acute membrane effects to occur. VA (n = 9) and VNA (n = 8) lactating rats were injected s.c with vehicle (12% ethanol in corn oil) at 20h and 2h prior to testing. All rats were transcardially perfused 90 min after the beginning of behavioural testing with 4% paraformaldehyde and the brains were collected and processed for Fos ICC (as described in Chapter 2). Fos expression was quantified in LS, BnST, MPOA, SON, MeA, CeA, PVN, PAG and OBs using the method described in chapter 2.

5.2.1.1 Statistics

For behavioural data, a T-test was performed when comparing the APA group with the VA group. The same was used to compare the VNA group with the APNA group. If data was not normally distributed, a Mann Whitney Rank Sum test was performed instead. For Fos data, a one way ANOVA was used to compare all groups. Data that was normally distributed were subjected to a Holm-Sidal post hoc analysis test. Data which were not normally distributed underwent a one way ANOVA on ranks followed by a Dunn's post hoc test. Data were deemed significant when $p \leq 0.05$.

5.2.2 Results

5.2.2.1 Aggressive behaviour

AP treatment had no significant effect on the number of attacks performed during a 30 min maternal aggression test ($p=0.67$, $t_{-0.4}$; Fig. 5.1). The average latency to attack

the intruder was also not significantly affected by AP treatment (p=0.33, $t_{1.0}$; Fig. 5.1). A comparison of the percentage of total time spent attacking the intruder between the vehicle and AP treated groups was carried out (Fig. 5.2). The data was subsequently analysed to either include sniffing behaviour or not because sniffing can be defined as both an aggressive behaviour and an investigative one. No significant difference was found between treatment groups when sniffing behaviour was included (p = 0.43, $t_{-0.8}$) or not (p = 0.78, $t_{-0.3}$).

5.2.2.2 Maternal behaviour

AP treatment had no significant effect on maternal behaviour expression between the aggression tested groups (p=1.0, $T_{(9,9)}$=85.0; Fig. 5.2). This was also the case between the non aggressive groups (p=0.74, $T_{(8,9)}$=68.0; Fig. 5.3).

5.2.2.3 General behaviour

The percentage of total time spent exhibiting general behaviour was also analysed and again AP had no significant effect on general behaviour expression compared to the vehicle treated group whether the group were aggression tested (p=0.86, $T_{(9,9)}$=83.0; Fig. 5.2) or not (p=0.74 $T_{(8,9)}$=76.0; Fig. 5.3).

5.2.2.4 Response to aggression behaviour by the resident

There was no significant effect of AP treatment on the percentage of total time spent exhibiting response to aggressive behaviour from the intruder by the resident compared to the vehicle treated group (p=0.72, $T_{(9,9)}$=90.0; Fig. 5.2).

5.2.2.5 Fos immunocytochemistry

In order to map the brain areas that were activated during the maternal aggression test or maternal behaviour observation, Fos immunocytochemistry was performed on the brains of the lactating rats. Specific brain regions, namely the LS, BnST, MPOA,

SON, MeA, CeA, PVN, PAG and OBs, already highlighted in previous studies (Meddle *et al*, unpublished, [12, 194, 196]) to be involved in maternal aggression and maternal behaviour were examined.

Fos expression was higher in the aggression tested groups (both vehicle and AP treated) in the BnST (VA vs VNA, $p=0.007$, $F_{(3,24)}=5.16$; APA vs VNA, APNA, $p=0.007$, $F_{(3,24)}=5.16$) and the MeA (VA vs VNA, APNA, $p<0.001$, $H_3=17.82$; APA vs VNA, APNA, $p<0.001$, $H_3=17.82$) than the non-aggression tested (both vehicle and AP treated) groups. In the OB, both vehicle and AP treated aggression tested groups had significantly higher Fos expression than the vehicle treated non-aggression tested group ($p<0.001$, $H_3=17.33$) but not the AP treated non-aggression tested group.

Interestingly in the PVN, Fos expression was significantly higher in the vehicle aggression tested group compared to all other groups ($p<0.001$ $F_{(3,26)}=14.77$). Similarly in the PAG, expression is highed in the vehicle aggression tested group compared to the AP treated aggression and non-aggression tested groups ($p=0.044$, $F_{(3,23)}=3.15$) but not the vehicle treated non-aggression tested group. However in the CeA, Fos expression is significantly higher in the AP treated aggression tested group compared to all other groups ($p<0.001$ $F_{(3,26)}=13.02$).

No significant difference was observed in Fos expression in the LS ($p=0.689$, $H_3=1.47$), SON ($p=0.084$, $H_3=6.64$) or MPOA ($p=0.090$ $H_3=6.5$) between any of the groups.

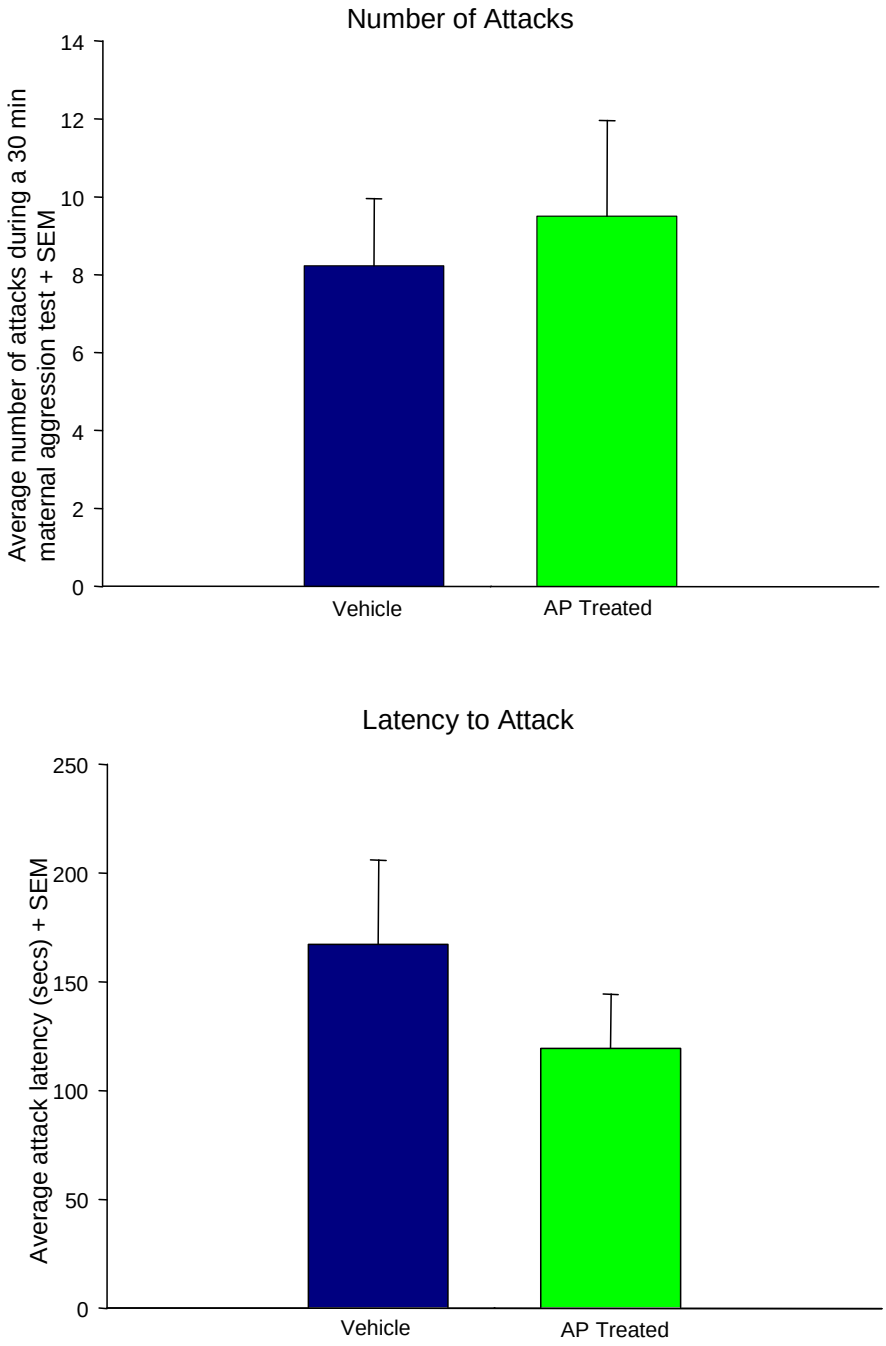


Figure 5.1: Average number and latency to attack by the resident lactating rat during a maternal aggression test following allopregnanolone treatment. A) The average number of attacks towards a novel virgin female intruder made by a resident lactating rat in her home cage with the pups present (vehicle, n=9; Allopregnanolone (AP) treated, n=8) during 30 min timed exposure. AP treated rats were injected with 3mg/kg (6mg AP in 12% ethanol and corn oil) and 1mg/kg (2mg AP in 12% ethanol and corn oil) of AP 20 and 2h respectively prior to behavioural testing. Vehicle treated rats were injected at the same time as AP treated rats with 12% ethanol and 88% corn oil. B) The average attack latency (secs) for resident lactating rat to attack an intruder in her home cage with her pups present during a 30 min maternal aggression test. Data are presented as the mean + SEM.

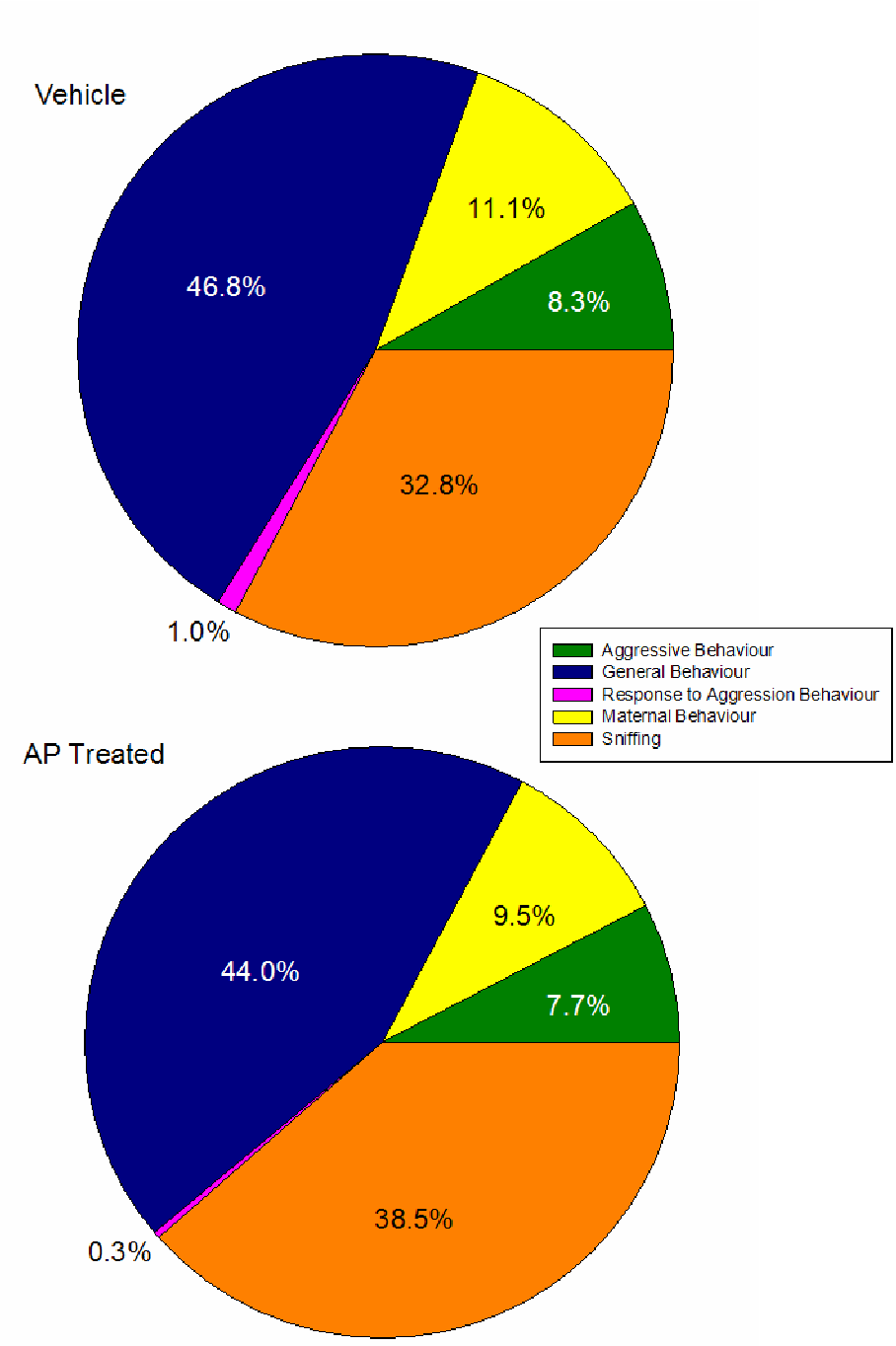


Figure 5.2: Average percentage of total time spent exhibiting different behaviours by resident lactating rat during a maternal aggression test following allopregnanolone treatment. The mean percentage of the total time (30 min) that resident lactating rats (vehicle, n=9; allopregnanolone (AP) treated, n=8) exhibited attacking, maternal, response to aggression, general or sniffing behaviours during exposure to a novel virgin female intruder in their home cage with their pups still present. AP treated rats were injected with 3mg/kg (6mg AP in 12% ethanol and corn oil) and 1mg/kg (2mg AP in 12% ethanol and corn oil) of AP 20 and 2h respectively prior to behavioural testing. Vehicle treated rats were injected at the same time as AP treated rats with 12% ethanol and 88% corn oil. There are no differences in percentage of total time spent exhibiting any behaviour between the two treatment groups.

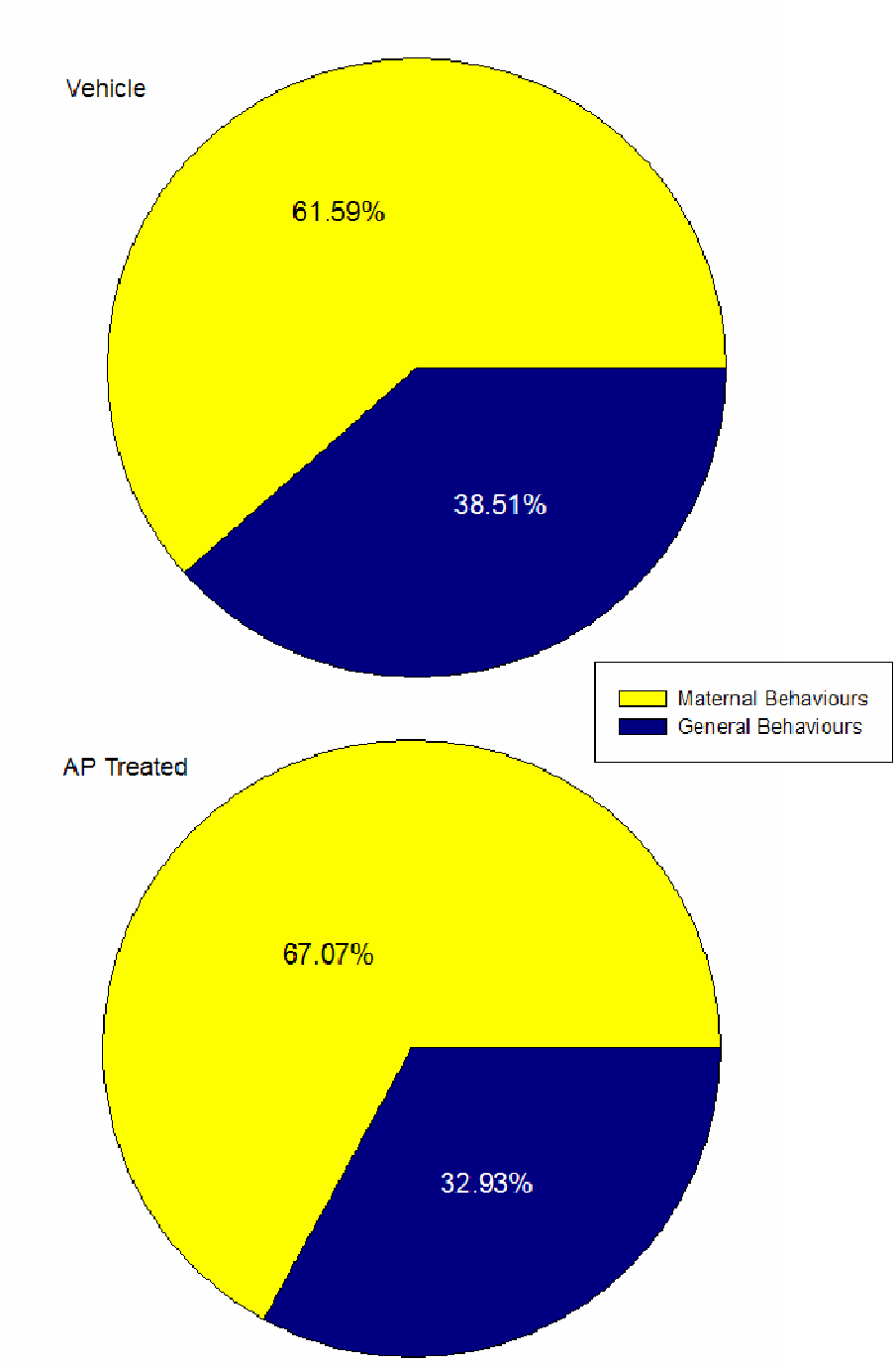


Figure 5.3: Average percentage of total time spent exhibiting different behaviours by the resident lactating rat during maternal behaviour observation following allopregnanolone treatment. The mean percentage of total time (30 min) that the resident lactating rat (vehicle, n=8; allopregnanolone (AP) treated, n=9) exhibited maternal or general behaviour in their home cage with their pups present. AP treated rats were injected with 3mg/kg (6mg AP in 12% ethanol and corn oil) and 1mg/kg (2mg AP in 12% ethanol and corn oil) of AP 20 and 2h respectively prior to behavioural testing. Vehicle treated rats were injected at the same time as AP treated rats with 12% ethanol and 88% corn oil. There is no difference in percentage of total spent exhibiting different behaviour between the two treatment groups.

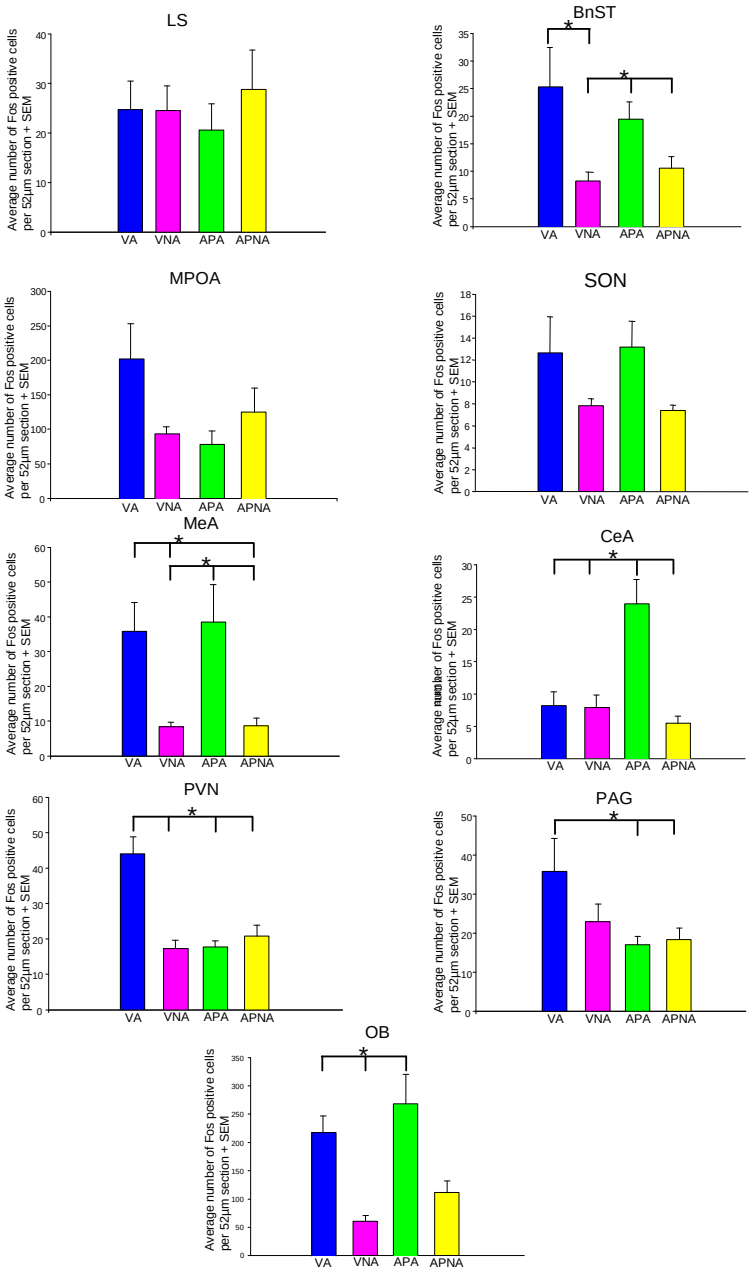


Figure 5.4: Average number of Fos positive cells in specific brain areas of the lactating rat following a maternal aggression test and allopregnanolone treatment. Allopregnanolone (AP) treated lactating rats were injected with 3mg/kg (6mg in 12% ethanol and 88% corn oil) and 1mg/kg (2mg in 12% ethanol and 88% corn oil) of AP 20 and 2h respectively prior to behavioural testing. Vehicle treated rats were injected at the same time as AP treated rats but with 12% ethanol and corn oil instead. Expression of Fos in the lateral septum (LS), bed nucleus of stria terminalis (BnST), medial preoptic area (MPOA), supraoptic nucleus (SON), medial and central amygdala (MeA and CeA respectively), paraventricular nucleus (PVN) , periaqueductal grey area (PAG) and olfactory bulbs (OBs) of AP or vehicle treated and aggression tested lactating rats (APA, n=8; VA, n=9) and AP or vehicle treated and non-aggression tested lactating rats (APNA, n=9; VNA, n=8) was examined using immunocytochemistry on brains perfused 90 min after the start of behavioural testing. Data are represented as mean + SEM. *=p≤0.05. A one way ANOVA was used to compare all groups followed Holm-Sidal post hoc analysis test. Data which were not normally distributed underwent a one way ANOVA on ranks followed by a Dunn's post hoc test.

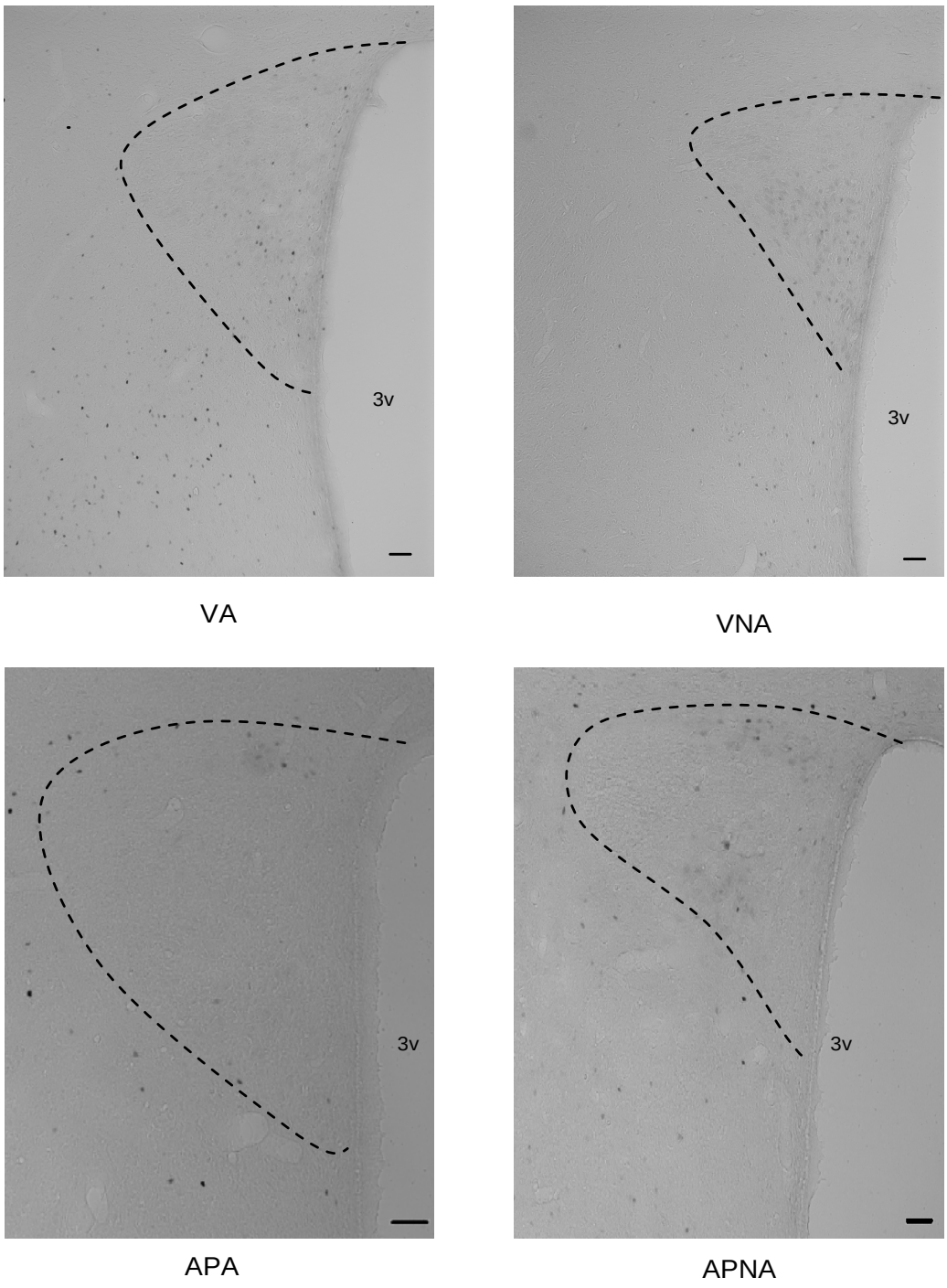


Figure 5.5: Photographs of Fos positive cells in the paraventricular nucleus of vehicle and allopregnanolone treated lactating rats after a maternal aggression test or maternal behaviour observation. Fos expression was examined in the paraventricular nucleus (defined by black dashed line) of allopregnanolone (AP) or vehicle treated and aggression tested lactating rats (APA, n=8; VA, n=9) and AP or vehicle treated and non-aggression tested lactating rats (APNA, n=9; VNA, n=8) using immunocytochemistry on brains perfused 90 min after the start of behavioural testing. AP treated rats were injected with 3mg/kg (6mg AP in 12% ethanol and corn oil) and 1mg/kg (2mg AP in 12% ethanol and corn oil) of AP 20 and 2h respectively prior to behavioural testing. Vehicle treated rats were injected at the same time as AP treated rats with 12% ethanol and 88% corn oil. Scale bars = 50μm. Abbreviations 3v= third ventricle.

5.3 The effect of allopregnanolone on pup retrieval behaviour

As AP treatment appeared to have no direct effects on the display of maternal aggression, expression of another component of maternal behaviour, specifically pup retrieval, was examined to observe if it was affected by AP treatment. This experiment was done with the assistance of student, Louise Warren.

5.3.1 Method

Lactating rats were injected s.c. 3mg/kg AP (Steraloids Inc. Ltd London; 6 mg AP in 1ml 12% ethanol in corn oil; n=10) or vehicle (12% ethanol in corn oil; n=9) 20h and again with 1mg/kg AP (2 mg AP in 1ml 12% ethanol in corn oil) or vehicle 2h prior to a pup retrieval task (see chapter 2 for detail).

5.3.1.1 Statistics

T-tests were performed to compare the two treatment groups for both behavioural and Fos data. Statistical significance was deemed when $p \leq 0.05$.

5.3.2 Results

5.3.2.1 Latency to approach a pup

AP treatment had no significant effect on the average latency to approach a pup ($p=0.84$, $t_{0.2}$; Fig. 5.7). For this analysis all behaviour relating to pups was included i.e. approach, lick, retrieve or move. Rats took on average 6.64±1.1sec (n=19) to approach the pup.

5.3.2.2 Latency to move a pup

For the analysis of average latency to move a pup, both moving the pups and pup retrieval were included. There was no significant effect of AP treatment on the average latency for a lactating rat to move a pup compared to the vehicle treated

group ($p=0.29$, $t_{-1.1}$). The vehicle treated group took 25.82±7.2sec to move a pup compared to 42.52±12.8sec for the AP treated group (Fig. 5.7).

5.3.2.3 Latency to retrieve pups

The latency to retrieve both first pup and fourth pup was analysed (Fig. 5.7). Retrieval was defined as the picking up of a pup (from a place other than the main nest), returning, and setting it down in the main nest. There was no significant effect of AP treatment to a lactating rat on the latency to retrieve either the first ($p=0.49$, $T_{(9,10)}=81.0$) or fourth pup ($p=0.60$, $T_{(9,9)}=79.0$) compared to the vehicle treated group.

5.3.2.4 Total time spent moving or retrieving pups

The percentage of total time spent moving and retrieving or just retrieving pups was analysed to investigate if there was any difference in the time taken to return the pup to the nest. Fig. 5.8 illustrates pie charts depicting percentage of total time exhibiting these different behaviours for the two treatment groups. There was no significant difference in the time spent moving and retrieving pups ($p=0.60$, $T_{(9,10)}=97.0$) or just retrieving pups ($p=0.60$, $T_{(9,10)}=97.0$) between the vehicle and AP treated groups.

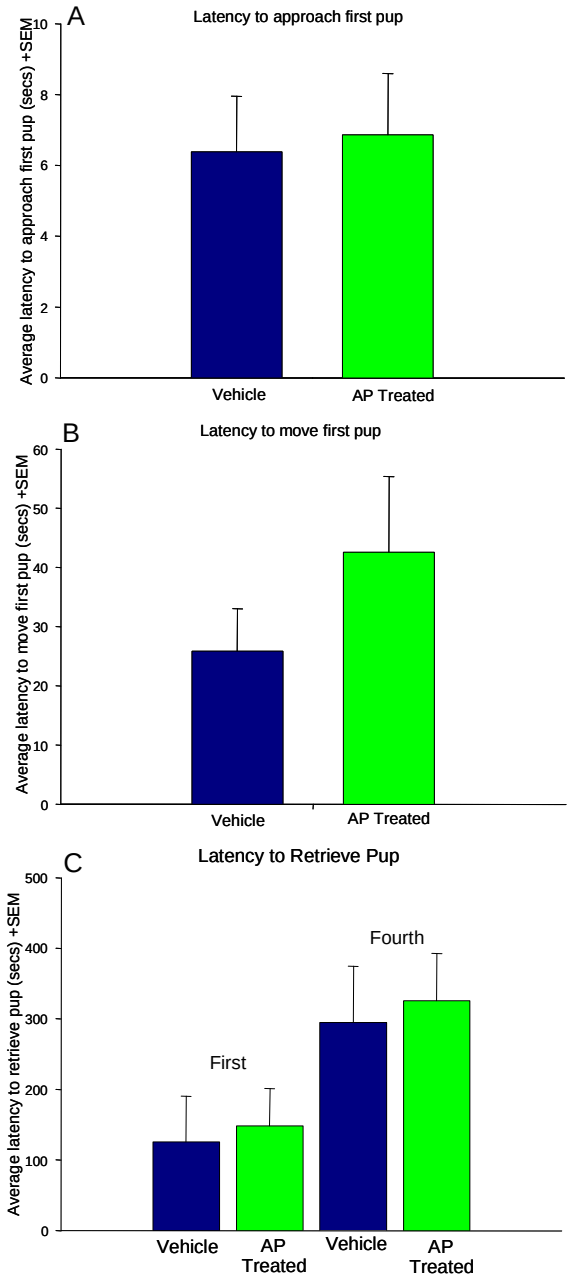


Figure 5.6: Average latency for the resident lactating rat to approach, move or retrieve pup during a pup retrieval task following allopregnanolone treatment. Allopregnanolone (AP) treated rats were injected with 3mg/kg (6mg AP in 12% ethanol and corn oil) and 1mg/kg (2mg AP in 12% ethanol and corn oil) of AP 20 and 2h respectively prior to behavioural testing. Vehicle treated rats were injected at the same time as AP treated rats with 12% ethanol and 88% corn oil. All pups were removed from the lactating resident rat and kept warm with bedding in a separate cage. After 1h of separation, 8 pups were randomly scattered around the home cage of the lactating rat and the behaviour digitally recorded until all pups were retrieved to the sleeping nest or 30 min had passed. The average time (secs) for the resident lactating rat (vehicle n=9, AP Treated n=10) to approach a pup in her home cage (A) or to pick up and carry a pup in her mouth (includes both retrieval and moving scored behaviours; B) or retrieve the first (C) and fourth pup (C) were examined. Retrieval was defined as the picking up of a pup (from area other than sleeping nest), carrying and placing the pup into the sleeping nest. Data are represented as mean + SEM.

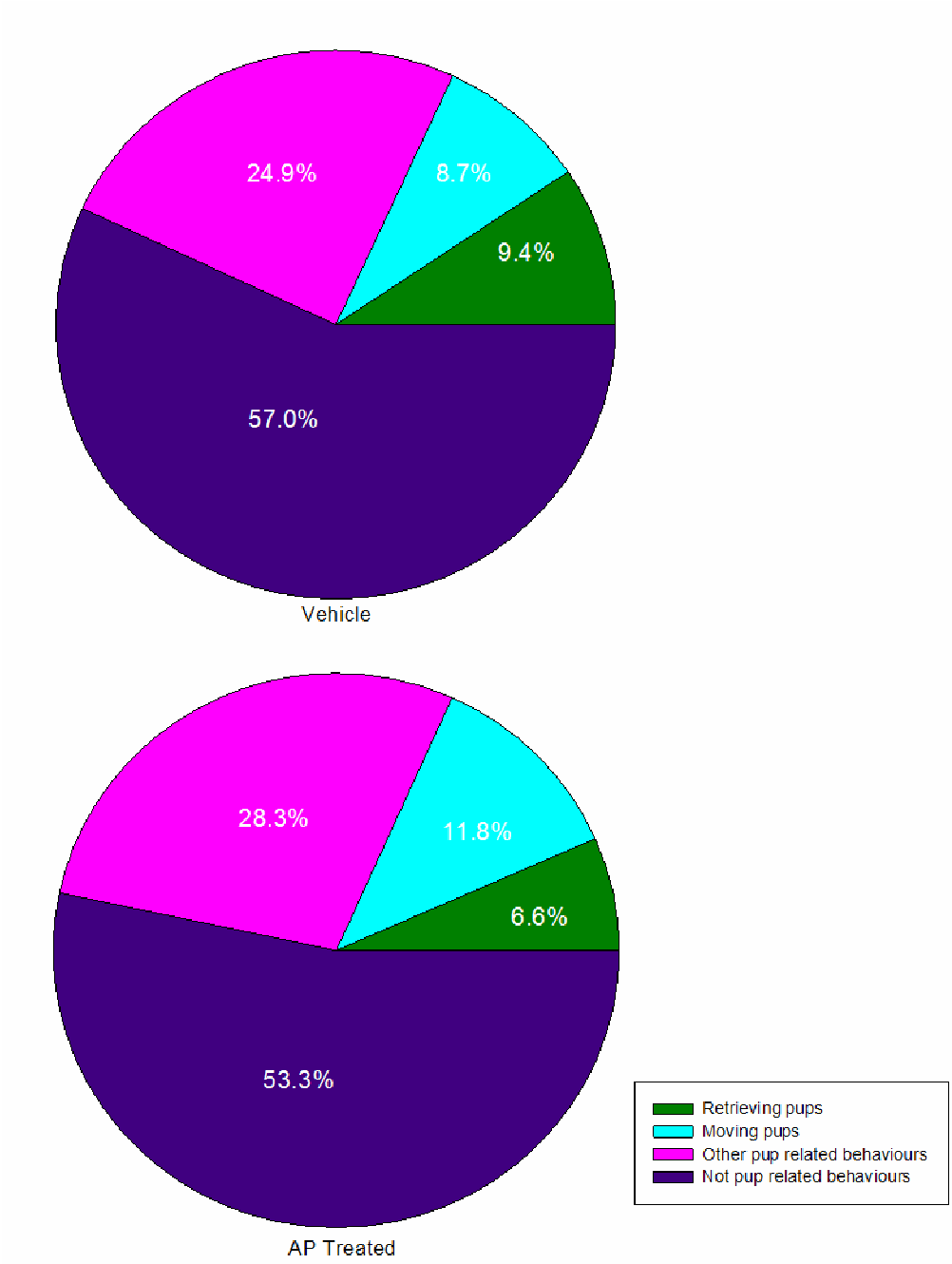


Figure 5.7: Average percentage of total time spent expressing different behaviours by the resident lactating rat during a pup retrieval task following allopregnanolone treatment. The mean percentage of total time (30 min) spent retrieving or moving pups or exhibiting other pup related or non pup related behaviours by a resident lactating rat (vehicle n=9, allopregnanolone (AP) treated n=10) during a pup retrieval task in her home cage. AP treated rats were injected with 3mg/kg (6mg AP in 12% ethanol and corn oil) and 1mg/kg (2mg AP in 12% ethanol and corn oil) of AP 20 and 2h respectively prior to behavioural testing. Vehicle treated rats were injected at the same time as AP treated rats with 12% ethanol and 88% corn oil. All pups were removed from the lactating resident rat and kept warm with bedding in a separate cage. After 1h of separation, 8 pups were randomly scattered around the home cage of the lactating rat and the behaviour digitally recorded until all pups were retrieved to the sleeping nest or 30 min had passed. There is no difference in percentage of total time spent exhibiting any behaviour between the two treatment groups.

5.4 The effect of maintaining allopregnanolone from late pregnancy until lactation on parturition and maternal aggression

The previous experiment examined the effect of AP administration during lactation on maternal behaviour, including maternal aggression and pup retrieval. Although no significant behavioural effects were observed, significant differences were observed in Fos expression in brains regions, such as the PVN, MPOA and CeA, suggesting that AP may have a regulatory effect on the stress axis and the fear and anxiety circuitry. Concas *et al* (1998, [346]) report the peak concentration of AP in the brain cortex is on day 19 of pregnancy, therefore this experiment aims to investigate whether maintaining AP levels from this time would impact firstly impact upon on parturition and secondly alter maternal behaviour including aggression expression. This experiment was done with the assistance of students, Gemma Rushton and Surawee Chuaiphichai.

5.4.1 Method

One day before expected parturition (pregnancy day 21), rats were s.c. injected with 3mg/kg AP (6 mg AP in 12% ethanol and corn oil; n=9) or vehicle (12% ethanol and 88% corn oil; n=10). On the morning of expected parturition, rats were injected using the same dose and route. Parturition was observed and digitally recorded with pup birth interval times noted. Following parturition, lactating rats were weighed and injected daily with the same dose between 9.00am and 11.00am. On lactation day 3, 2h prior to a 10 min maternal aggression test lactating rats were injected s.c. with 1mg/kg AP (2 mg AP in 12% ethanol and corn oil) or vehicle injection. Rats were perfused 90 min following the start of the maternal aggression test and the brains

collected for Fos ICC. Fos was examined in the LS, BnST, MPOA, SON, MeA, CeA, PVN and PAG using the technique described in chapter 2.

5.4.1.1 Statistics

A two way ANOVA was performed comparing treatment and pup birth interval times. A T-test was used to compare behavioural and Fos data between the treatment groups. When $p \leq 0.05$, data was deemed to be statistically different.

5.4.2 Results

5.4.2.1 Pup birth interval

AP administration prior to parturition had no significant effect on the timing of parturition onset ($p=0.65$, $T_{(7,8)}=86.0$) or pup birth interval ($p=0.789$, $F_{(1,121)}=0.0718$; Fig. 5.9) compared to vehicle treatment.

5.4.2.2 Aggressive behaviour

Maintaining elevated levels of AP from late pregnancy resulted in significantly more attacks ($p=0.045$, $T_{(8,10)}=99.0$; AP=8.25±2.1 attacks in a 10 min maternal aggression test, V=3.60±.5; Fig. 5.10) but there was no significant effect on the latency to attack ($p=0.25$, $t_{-1.2}$; AP=95.75±27.3sec, V=168.82±49.9sec; Fig. 5.10) or percentage of total time exhibiting aggressive behaviour ($p=0.27$, $T_{(9,10)}=104.0$; Fig. 5.11) compared to vehicle treatment. When individual aggressive behaviours are compared between the two treatment groups as a percentage of total aggressive behaviour time, no statistical difference is found in any type of behaviour (attacks $p=0.93$, $t_{-0.08}$, biting $p=0.49$, $T_{(9,10)}=81.0$, clawing $p=0.22$, $T_{(9,10)}=105.0$, rearing $p=0.13$, $T_{(9,10)}=71.0$, pinning down $p=0.41$, $t_{0.8}$ and lunging $p=0.84$, $T_{(9,10)}=93.0$; Fig. 5.12).

5.4.2.3 Maternal behaviour

No significant effect of AP treatment in time spent exhibiting any maternal behaviour (all maternal behaviours p=0.84, $T_{(9,10)}$=87.0, nursing p=0.41, $T_{(9,10)}$=79.5, general pup interaction p=0.60, $T_{(9,10)}$=83.0; Fig. 5.11) compared to vehicle treatment.

5.4.2.4 General behaviour

When all general behaviour was analysed as a percentage of total time, there no significant effect of AP treatment compared to vehicle (p=0.23, $t_{-1.2}$; Fig. 5.11). However, when general behaviour was broken down into its components and compared as a percentage of total general time. The AP treated group spent significantly more time than the vehicle treated group performing general behaviours (p=0.025, $t_{2.5}$; Fig. 5.13) and significantly less time grooming themselves (p=0.005, $t_{-3.2}$; Fig. 5.13) than the vehicle treated group.

5.4.2.5 Response to aggression behaviour by the resident

There was no significant effect of AP treatment in the behavioural response of the resident to intruder aggressive behaviour when compared as a percentage of total time (p=0.59, $T_{(9,10)}$=97.0) or when broken down into its individual components (freezing p=0.84, $T_{(9,10)}$=93.0, escaping p=0.97, $T_{(9,10)}$=90.0 or rearing away p=0.48, $T_{(9,10)}$=99.0; Fig. 5.11) compared to vehicle treatment.

5.4.2.6 Fos immunocytochemistry

AP treatment significantly reduced Fos expression in the BnST of aggressive rats compared to the vehicle treated group (p=0.020, $t_{-2.6}$; Fig. 5.14). No significant difference in Fos expression was observed in the LS (p=0.099, t=1.7), CeA (p=1.0, $T_{(8,10)}$=75.5), MeA (p=0.16, $t_{-1.5}$), MPOA (p=0.89, T=74.0), SON (p=0.22, $t_{-1.7}$),

parvocellular PVN ($p=0.89$, $T_{(8,9)}=70.0$) or PAG ($p=0.31$, $T_{(9,10)}=103.0$; Fig. 5.14) between the AP and vehicle treated groups. There were not enough sections containing magnocellular PVN areas per rat to be able to perform statistical analysis.

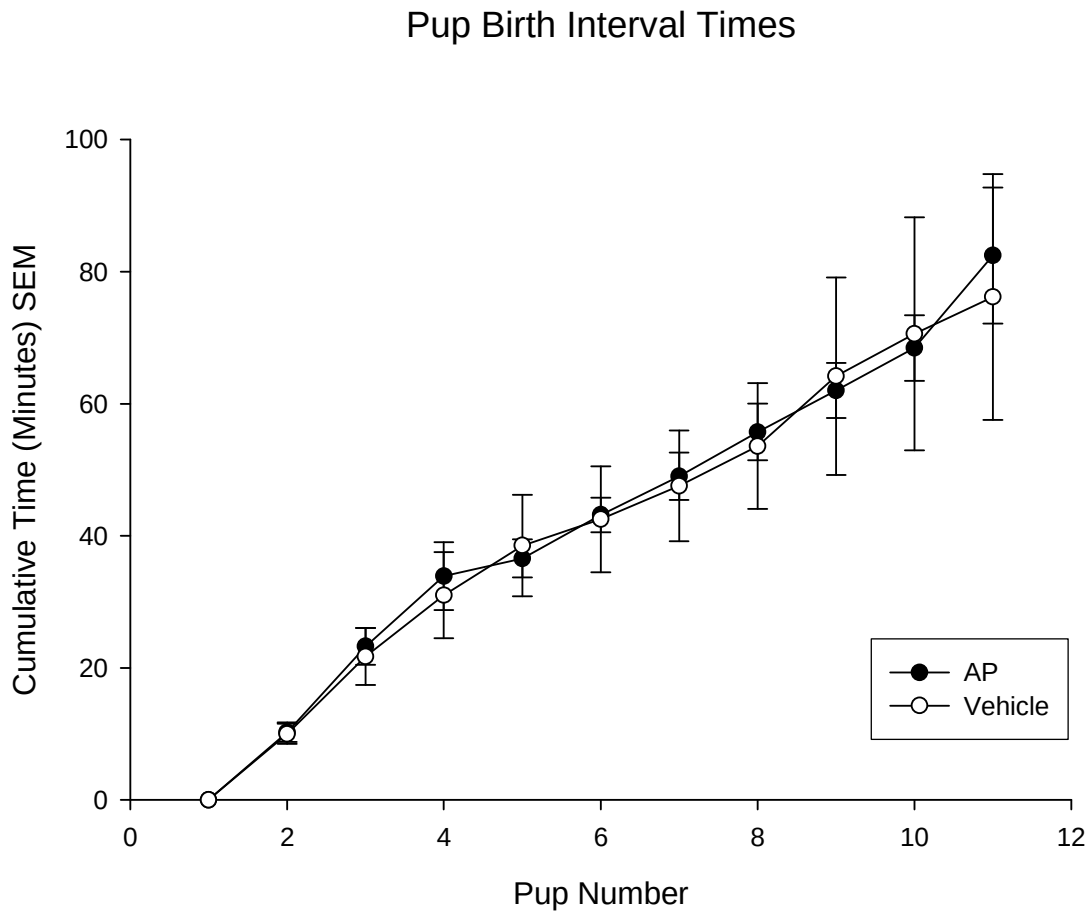


Figure 5.8: Effect of allopregnanolone administration on pup birth interval latency during parturition. Pregnant rats were subcutaneously injected with allopregnanolone (AP; 6mg AP in 12% ethanol and corn oil; n=9) or vehicle (12% ethanol and 88% corn oil; n=10) one day prior (deemed 21 days after seminal plug found) and on the day of parturition. The time (min) between the birth of each pup was recorded and is represented here as cumulative time ± SEM for the first 11 pups. p=0.79, F (1,121)=0.0718; 2,way ANOVA.

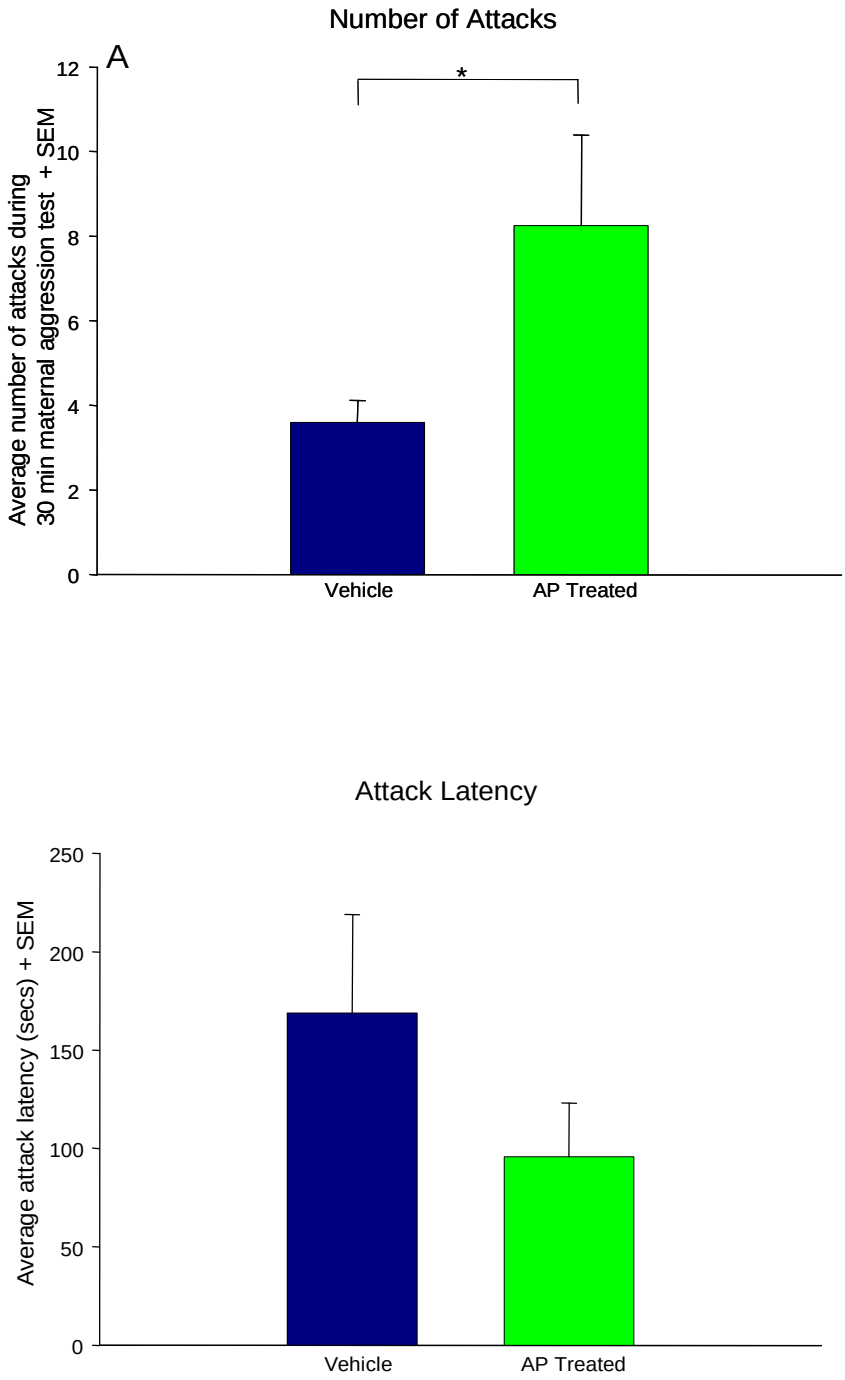


Figure 5.9: Average number of attacks and attack latency for a resident lactating rat during a maternal aggression test following allopregnanolone treatment. The allopregnanolone (AP; n=9) group was treated daily with subcutaneous injections of 3mg/kg AP (6mg of AP in 120µl corn oil and 880µl ethanol (100%)) from one day prior to parturition until one day prior to testing. They also received an injection of 1mg/kg AP 2h prior to maternal aggression test. The vehicle group (n=10) was also injected at the same times as the AP with 12% corn oil and 88% ethanol. (A) Average number of attacks by a resident rat during a 10 min maternal aggression test towards a novel virgin female intruder in their home cage with their pups present. (B) The mean latency (secs) to attack an intruder by the resident lactating rat during a 10 min maternal aggression test. Data are represented as mean +SEM. *=p≤0.05

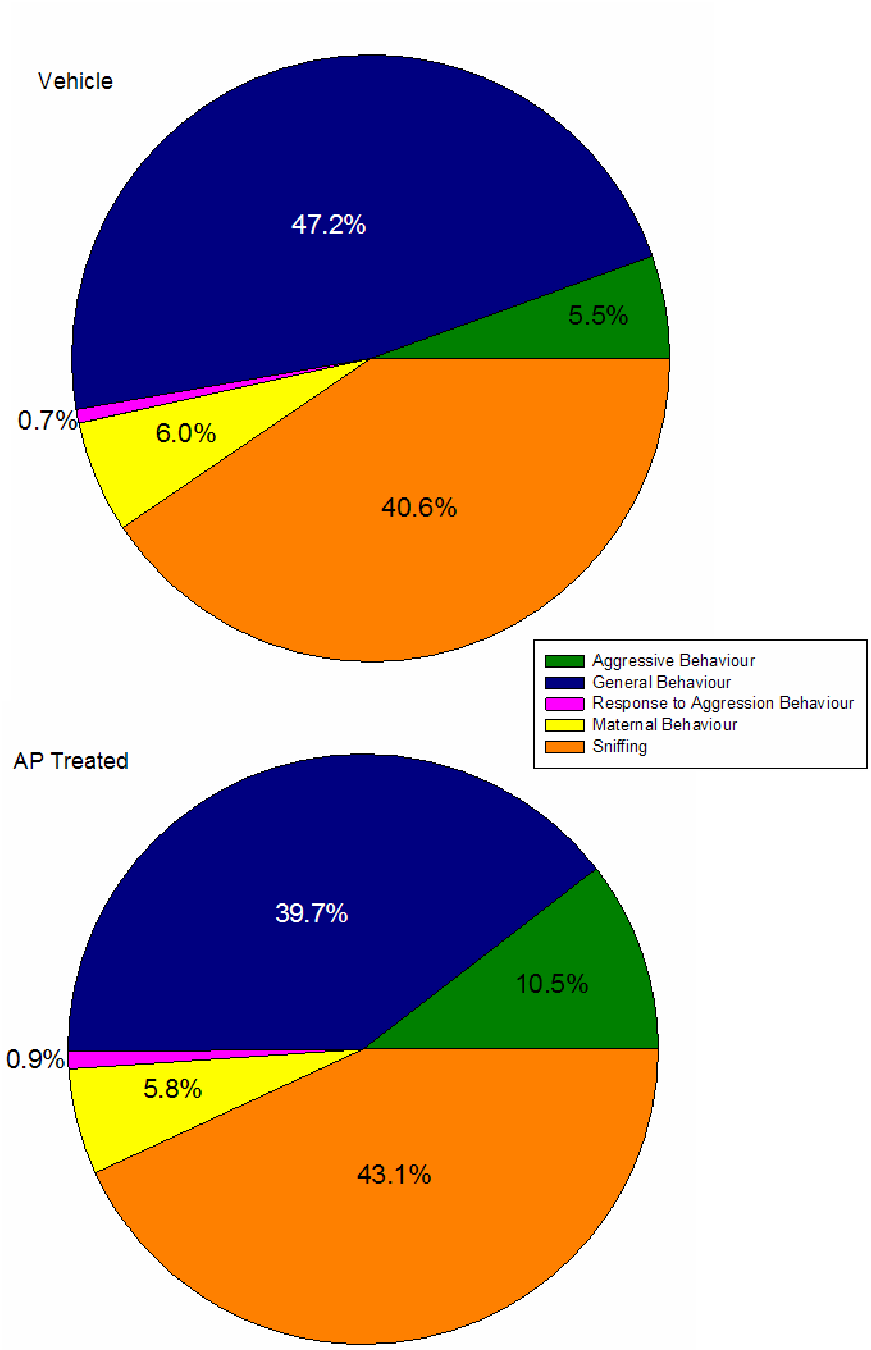


Figure 5.10: Average percentage of total time spent exhibiting different behaviours by resident rat during a maternal aggression test following allopregnanolone treatment. The allopregnanolone (AP; n=9) group was treated daily with subcutaneous injections of 3mg/kg AP (6mg of AP in 120µl corn oil and 880µl ethanol (100%)) from one day prior to parturition until one day prior to testing. They also received an injection of 1mg/kg AP 2h prior to maternal aggression test. The vehicle group (n=10) was also injected at the same times as the AP with 12% corn oil and 88% ethanol. The mean percentage of total time (10 min) spent exhibiting attacking, maternal, general, response to aggression or sniffing behaviours by a resident lactating rat in her home cage with her pups present during maternal aggression test. There was no significant difference in the display of any behaviour between the two treatment groups.

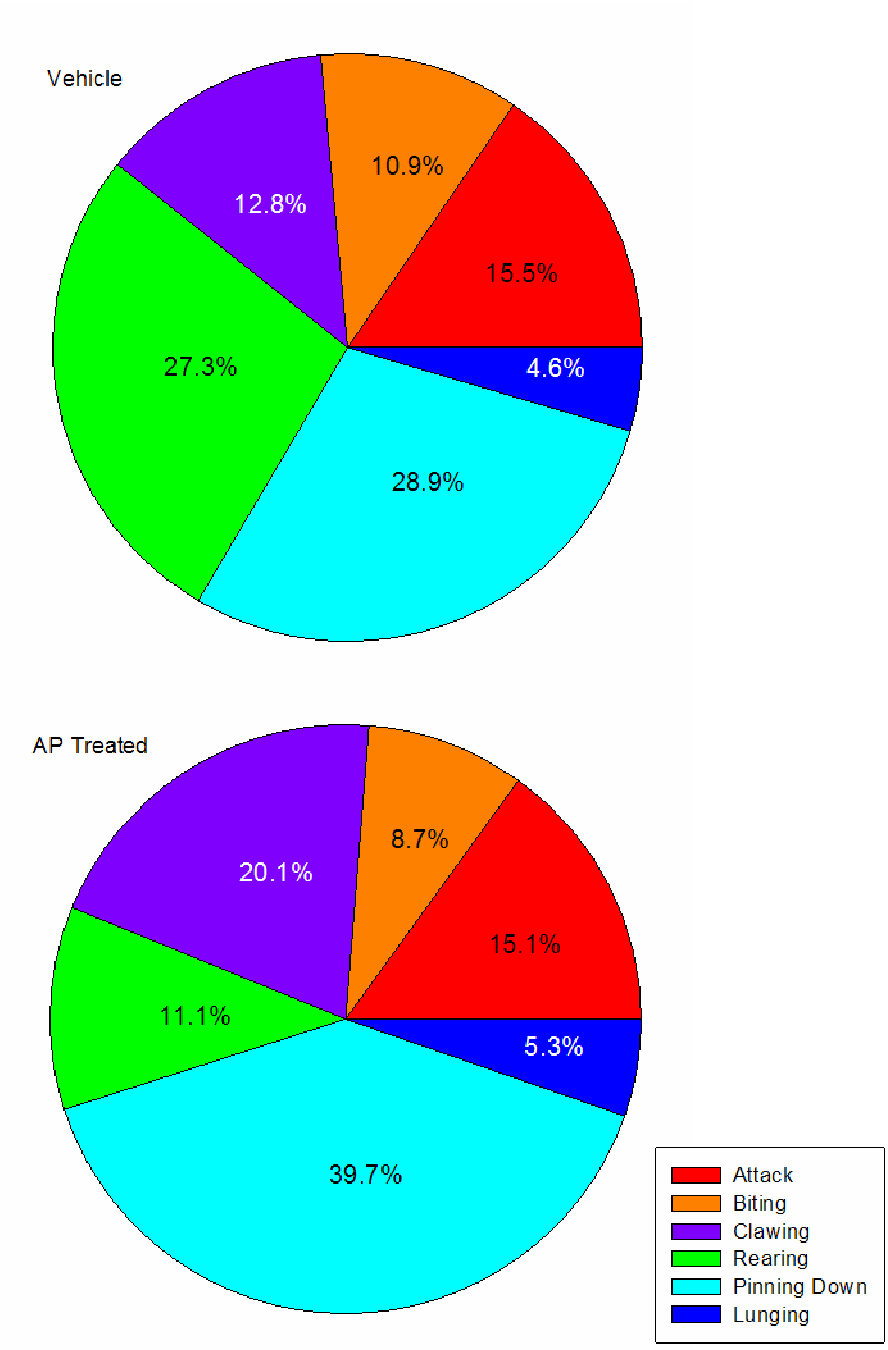


Figure 5.11: Average percentage of total aggression time spent exhibiting different aggressive behaviours by a resident rat during a maternal aggression test following allopregnanolone treatment. The allopregnanolone (AP; n=9) group was treated daily with subcutaneous injections of 3mg/kg AP (6mg of AP in 120µl corn oil and 880µl ethanol (100%)) from one day prior to parturition until one day prior to testing. They also received an injection of 1mg/kg AP 2h prior to maternal aggression test. The vehicle group (n=10) was also injected at the same times as the AP with 12% corn oil and 88% ethanol. Average percentage of total aggression time spent exhibiting attacking, biting, clawing, rearing, pinning down and lunging behaviour towards a novel virgin female intruder during a 10 min maternal aggression test by a lactating rat in her home cage with her pups present. No significant differences are observed in the expression of any aggressive behaviour between the two treatment groups.

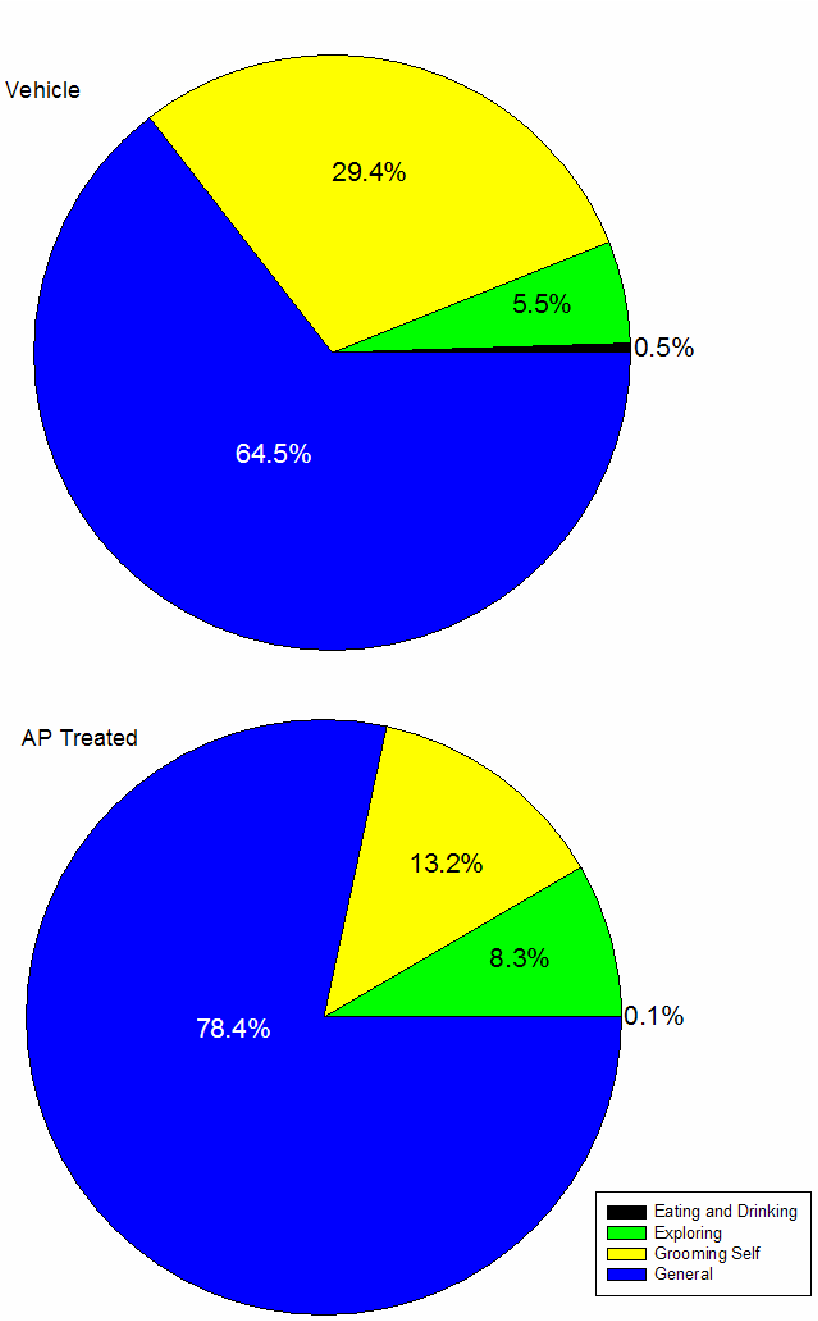


Figure 5.12: Average percentage of total general time spent exhibiting different general behaviours by a resident rat during a maternal aggression test following allopregnanolone treatment. The allopregnanolone (AP; n=9) group was treated daily with subcutaneous injections of 3mg/kg AP (6mg of AP in 120µl corn oil and 880µl ethanol (100%)) from one day prior to parturition until one day prior to testing. They also received an injection of 1mg/kg AP 2h prior to maternal aggression test. The vehicle group (n=10) was also injected at the same times as the AP with 12% corn oil and 88% ethanol. The average percentage of total general time spent exhibiting eating/drinking, exploring, grooming self or general (defined as walking around cage without interaction with pups or intruder) by the resident lactating rat during a 10 min maternal aggression test with her pups present. Note that the AP treated group spend significantly more time grooming themselves and expression general behaviours than the vehicle treated group

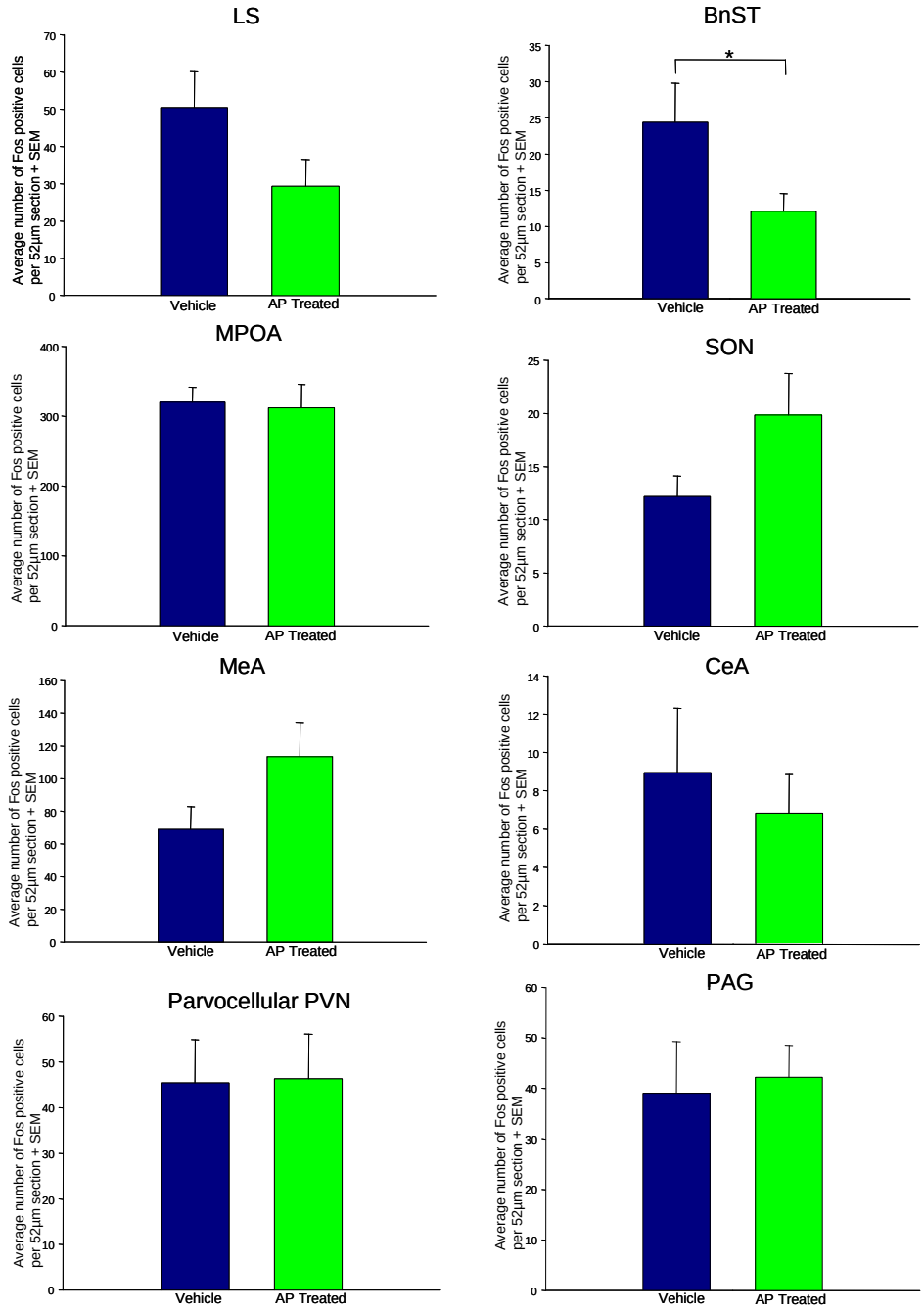


Figure 5.13: Average number of Fos positive cells in specific brain areas of the resident lactating rat brain during a maternal aggression test following allopregnanolone treatment. Allopregnanolone (AP; n=9) treated rats were injected s.c. with 3mg/kg AP (6mg AP in 12% ethanol and 88% corn oil) daily from pregnancy day 21 until one day prior to behavioural testing. On the day of testing, rats received a 1mg/kg AP (2mg AP in 12% ethanol and 88% corn oil) injection s.c. 2h prior to a maternal aggression test. Vehicle treated rats (n=10) were injected at the same time as AP treated rats but with 12% ethanol and corn oil. Expression of Fos was examined in the lateral septum (LS), bed nucleus of stria terminalis (BnST), medial preoptic area (MPOA), supraoptic nucleus (SON), medial and central amygdala (MeA and CeA respectively), parvocellular paraventricular nucleus (PVN) and periaqueductal grey area (PAG) of AP or vehicle treated lactating rats by performing immunocytochemistry for Fos on brains perfused 90 min after the start of behavioural testing. Data are represented by means + SEM. *p≤0.05

5.5 The effect of finasteride (5α reductase inhibitor) treatment from late pregnancy on maternal aggression

In the previous experiments, levels of AP were manipulated during lactation or prevented from decreasing from late pregnancy onwards. The number of attacks was significantly increased but no other behavioural measurements were affected when AP levels were maintained from late pregnancy. There is a possibility that AP does have a positive modulatory effect on maternal aggression but quantification of the behaviour in this study was not sensitive enough to detect the differences. It is also possible that a capping effect may be occurring as the behavioural test was performed on days 3 to 7 of lactation when the level of aggression is at its highest expression. Therefore in this study the aim was to examine the effect of administering finasteride, a 5α reductase inhibitor (the rate limiting step in the production of AP), daily from late pregnancy, prior to the peak in AP (pregnancy day 19; [346]) until lactation day 3 on maternal aggression expression.

5.5.1 Method

Daily injections of 50mg/kg of finasteride (50mg in 234µl of ethanol with 766µl of corn oil; n=7) or vehicle (234µl of ethanol with 766µl of corn oil; n=11) was initiated on pregnancy day (PD) 17 (because AP levels in the cerebral cortex are reported to be at their peak in rats at PD19 [346]). This dosage of finasteride was used because it was already observed to cause significant changes in behaviour including maternal within female rats [368, 451, 455-458]. Immediately prior to the first injection on PD17, a blood sample (200µl) was taken from rats by tail tipping (chapter 2). To investigate if finasteride had any effect on parturition and lactation, pup birth intervals were recorded and the pups weighed daily following birth. The finasteride

injections were given daily until day 2 of lactation. On the day of behavioural testing (lactation day 3), rats were exposed to a 30 min maternal aggression test in their home cage with their pups present. A lethal overdose of sodium pentobarbitone (1ml) was given 90 min following the start of the test and a blood sample (200µl) was collected from the heart with heparinised capillary tubes. The blood samples were later processed for progesterone by RIA and compared to pre-finasteride levels to ensure that finasteride treatment has no effect on circulating progesterone levels.

5.5.1.1 Statistics

A t-test was used to compare the results between the two treatment groups, when $p \leq 0.05$ data were considered to be statistically significant.

5.5.2 Results

5.5.2.1 Aggressive behaviour

Finasteride treatment had no significant effect on aggressive behaviour. The number of attacks ($p=0.87$, $t_{-0.2}$; Fig. 5.15) and attack latency ($p=0.18$, $t_{-1.4}$; Fig. 5.15) were not statistically different between the two treatment groups. The percentage of total time spent exhibiting all aggressive behaviours was not significantly different between the two treatment groups ($p=0.11$, $T_{(7,9)}=75.0$; Fig. 5.15).

5.5.2.2 Other behaviours

Finasteride treatment had no significant effect on maternal ($p=0.94$, $t_{-0.07}$; Fig. 5.15) or general behaviour ($p=0.35$, $t_{-1.0}$; Fig. 5.15) expressed during a maternal aggression test compared to vehicle treatment. However, the finasteride treated group appeared more anxious as they spent significantly more time freezing in response to aggressive behaviour from the intruder compared to the vehicle treated group ($p=0.044$, $T_{(7,9)}=79.0$; Fig. 5.15).

5.5.2.3 Circulating progesterone levels

The levels of circulating progesterone did not significantly differ between treatment groups either on pregnancy day 17 (before the start of treatment; $p=0.20$, $t_{1.3}$; finasteride=75.62±3.2ng/ml, vehicle=67.65±5.0ng/ml) or on lactation day 3 following finasteride treatment (day of behavioural testing; $p=0.13$, $t_{1.6}$; finasteride=18.92±3.0ng/ml, vehicle=26.29±3.4ng/ml).

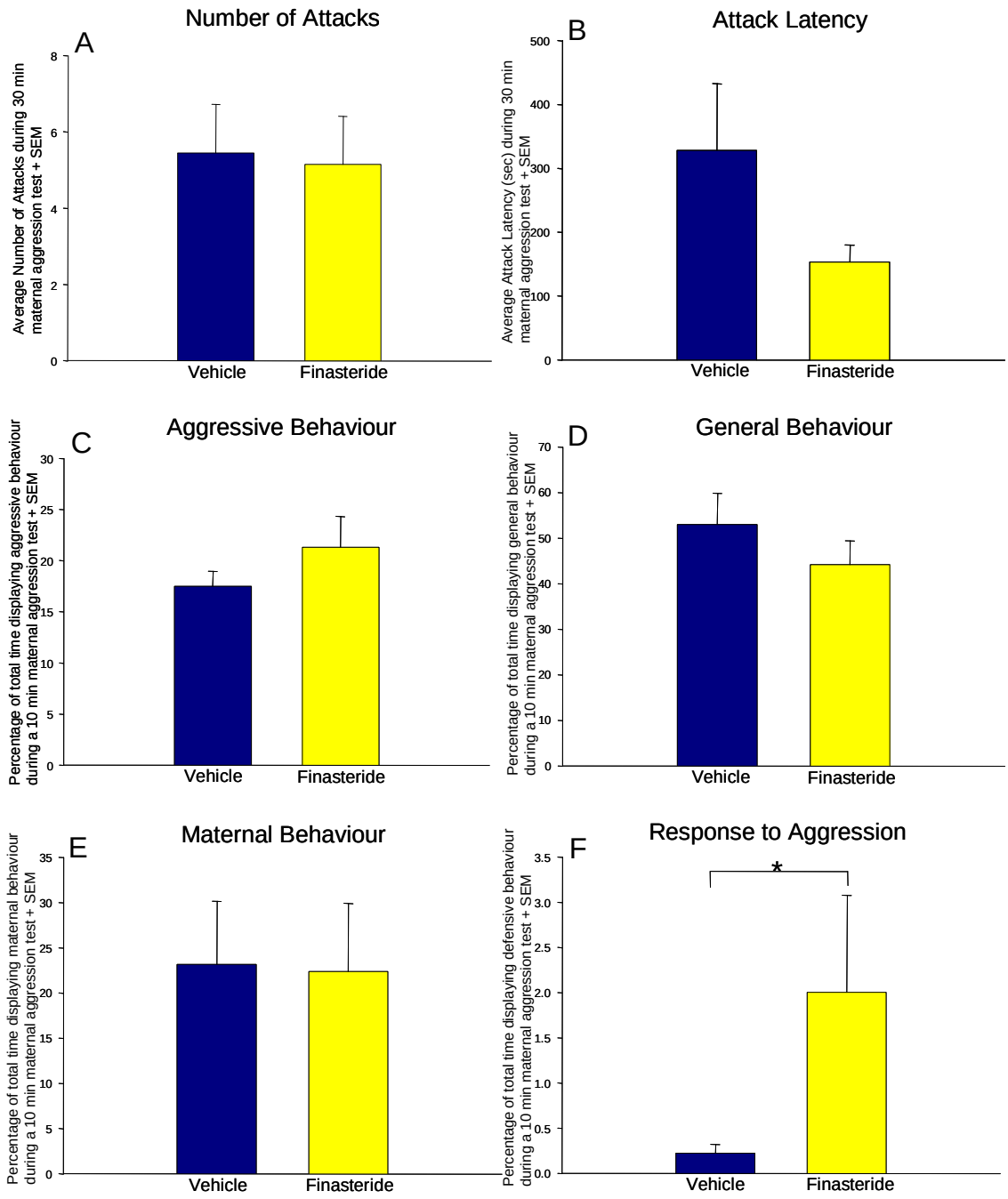


Figure 5.14: Average number of attacks, attack latency and percentage of total time displaying different behaviours for a resident lactating rat during a maternal aggression test following finasteride treatment. The finasteride (n=7) group was treated daily with subcutaneous injections of 50mg/kg finasteride (50mg of finasteride in 800µl corn oil and 200µl ethanol (100%)) from pregnancy day 17 until one day prior to testing. The vehicle group (n=11) was also injected at the same times as the finasteride group but with 80% corn oil and 20% ethanol instead. Average number of attacks (A) and latency to attack (B) by a resident lactating rat during a 10 min maternal aggression test towards a novel virgin female intruder in her home cage with her pups present. The percentage of total time expressing aggressive, general, maternal or response to aggression (C-F respectively) behaviour were also compared between the two treatment groups. Data are represented as mean + SEM. *=p≤0.05

5.6 Discussion

The results of this study firstly suggest the AP does not play a crucial role in the control of parturition. AP administered in late pregnancy did not have any effect on parturition onset or pup birth interval times. This was also the case if finasteride was administered from late pregnancy. Previous reports have shown progesterone to delay or even inhibit parturition in rats [459, 460]. Together these results indicate that central progesterone actions in late pregnancy are on its own receptor and not through AP inducing $GABA_A$ receptor inhibition of OXT cells [461]. AP is observed to significantly inhibit uterine contractibility directly through its actions on the $GABA_A$ receptor but no effect was observed in the present study suggesting the dosage administered is not enough to cause this effect [462].

Secondly the results show that AP does not exert a direct effect on maternal behaviour and aggression. Instead, AP may indirectly help enable maternal aggression by modulating fear and anxiety. Lactating rats are observed to display a lower anxiety and fear behaviour profile on the EPM, open field and defensive burying tests [31-33]. This reduction in fear and anxiety during lactation is thought to be essential for normal maternal behaviour, because virgin female rats are fearful of pups and do not spontaneously display maternal behaviour [29]. A significant increase in Fos expression was detected in the CeA following AP treatment in aggression tested rats, a brain region important in fear regulation [105]. It has even been suggested that during the peri-partum period GABA transmission increases in this area to inhibit the CeA projections to fear-inducing nuclei [265, 440]. Thus, it may be hypothesised that AP acts by enhancing GABA actions to reduce fear and anxiety so that the dam may display aggression.

Although no effect of AP administration during lactation was observed on maternal aggression, maintaining AP levels from late pregnancy did result in a significant increase in the number of attacks. Furthermore, this AP treatment resulted in a significantly higher level of Fos expression in the BnST compared to vehicle treated rats. The BnST is implicated in regulating the long term effects of anxiety whereas CeA mediates the short term (i.e. fear) [105].

Finally when finasteride, a 5α-reductase inhibitor (the rate limiting enzyme in AP production), was administered from late pregnancy (although no difference was observed in aggressive, maternal or general behaviour expression) it caused an increase in the amount of time freezing (an expression of fear) during an aggression test by the lactating rat. This supports the idea that AP indirectly helps maintain maternal aggression by regulating fear and anxiety to low levels.

AP potentiates $GABA_A$ receptor activation and has anxiolytic, anticonvulsant and neuroendocrine effects similar to those produced by benzodiazepine [31, 463]. If diazepam, a prototypical benzodiazepine, is administered to a female rat in proestrous, there is a detectable reduction in anxiety levels compared to ovariectomised females [31, 464]. Also, finasteride administration to postpartum rats increases depressive behaviour, such as forced swim test immobility similar to the increase in freezing seen in the maternal aggression test, in conjunction with decreased plasma and hippocampal AP levels [447]. However in the study by Picazo *et al* (1993, [33]) although they found anxiety was reduced in the defensive burying paradigm immediately after parturition, plasma progesterone levels were at their lowest at this time. Nonetheless AP can also be derived from cholesterol within the brain so circulating levels of progesterone may be irrelevant [32, 33].

Yet, the sensitivity of $GABA_A$ receptors is reported not to be altered in the lactating rat compared to a non-lactating cycling rat in response to either GABA or AP and MK404, another 5-α reductase inhibitor, administration. Also, the production of AP from progesterone in the serum was decreased in lactating rats but there was no observed effect on EPM performance [32]. However these rats were tested on lactation day 7 when anxiety levels are already increasing. Bitran *et al* (1991, [31]) had found greatest reduction in anxiety to be on lactation day 2. Furthermore, although AP production from progesterone was decreased in the serum, the level of AP in serum was still greater than non-lactating cycling female rats [32]. Also, there is a problem in using the EPM to measure anxiety in lactating rats as the observed increase in open arm exploration may in fact reflect increased vigilance or searching for pups [32].

However, although finasteride treatment increased freezing behaviour, an expression of 'fear-like' behaviour, in lactating rats during a maternal aggression test, there was no change in the level of aggression. Pup stimulation during the maternal aggression test may have helped the lactating rat overcome the increase in fear and hence still express aggression at normal levels [188]. Hence, further research investigating how the modulation of fear and anxiety is regulated by AP during lactation is warranted.

The reduction in anxiety behaviour observed during lactation is also detected during pregnancy where it has been linked with AP. Pregnancy day 19 rats spend more time on, and make more entries into, the open arms of the EPM compared to non-pregnant ovariectomised rats [34]. Anxiety is also reduced in the defensive burying paradigm in pregnant rats where an increase in burying behaviour latency

(pregnancy day 21) and a decrease in cumulative burying behaviour (pregnancy day 14) compared to ovariectomised rats was observed [33]. It has been characterized that plasma and cortical AP levels are at their highest during pregnancy [34, 346, 458]. Finasteride administration during pregnancy (day 19) to reduce AP levels comparable to those of oestrus significantly increases anxiety on the EPM [34].

Finasteride treatment has other significant effects during pregnancy, particularly in relation to the HPA axis. During late pregnancy, the HPA axis is normally inhibited by endogenous opioids induced by AP. By blocking AP production, finasteride removes this inhibition and the HPA axis responds to stressors including interleukin-1ß which mimics infection [199]. A reduced stress response during late pregnancy is important and is hypothesised to prevent chronic activation and therefore release of corticosteroids which can cross the mother-fetal barrier. High levels of glucocorticoids are known to be harmful to fetal development including the development of the limbic and neuroendocrine systems [34, 36]. AP could exert its protective control over the HPA axis by enhancing $GABA_A$ receptor inhibitory activity. This altered HPA axis activity is also observed in lactating rats in response to pup stimulation, suggesting that AP may also be important in regulating HPA axis activity during lactation, which could indirectly impact upon maternal behaviour and aggression [36].

The results from this current study support this idea as decreased Fos activation was observed in the PVN and PAG following AP treatment in aggression tested rats. The PVN is an area important in the control of the stress response as it is a crucial part of the HPA axis [107, 200, 201]. The reduction in Fos expression observed in the PAG again links the actions of AP with the stress response as the

PAG has a substantial role in determining what behaviour is expressed in reaction to a stressor [141]. The PAG also has a role in the control of other important maternal behaviours especially nursing [140].

It is important to note that AP treatment in the present study appeared to only have minimal effects on maternal behaviour and aggression. There are several possible explanations for this. One possibility is that the behavioural test may not be sensitive enough to detect the differences. Another possibility is that a capping effect may be occurring. The behavioural testing is performed during lactation days 3 to 7 when maternal aggression is at its highest, therefore if AP has a positive effect on maternal aggression it will cause little or no change.

It could be argued that our AP dosage was too low, being in the range that has been seen to have no effect on other forms of aggression [342, 379]. However, the dosage used in the current study is known to influence the actions of $GABA_A$ receptors on OXT release in the rat. The dosage was given to virgin female rats where it significantly lessened the OXT secretory response to an interleukin challenge, leading to the proposal that $GABA_A$ receptors on OXT neurons can be influenced by AP [199]. Also, administration of 1mg of AP subcutaneously prevented the rise in vasopressin mRNA in the PVN of adrenalectomised rats [446]. Hence, if progesterone does modulate maternal behaviour through the actions of AP on $GABA_A$ receptors, the dosage used is known to cause effects within the brain and therefore would allow for a difference in behaviour to be expressed. Indeed, the Fos data show that AP has an effect in the brain but not on behaviour as significant differences were observed in AP treated compared to vehicle treated lactating rats.

In humans, anxiolytic effects of AP are beginning to be discovered, especially in relation to hormonal cycles, pregnancy and the post partum period. In both humans and rodents, AP levels rise throughout pregnancy to create an anxiolytic effect so pregnancy can reach term with little deleterious damage to the fetus [465]. During pregnancy, women display a stable psychological status, which is hypothesized to be due to the increase in the anti-anxiety neurosteroid AP levels [453]. It has been postulated that the rapid decline in the levels of AP around the end of pregnancy may contribute to the development of postpartum depression [441]. The decrease in AP levels, both blood and brain, have been found to impact upon many mood and anxiety disorders, including premenstrual tension (PMT) and pregnancy related depression which are hormone related [463]. For example, Montelone *et al* (2000, [466]) noticed that sufferers of PMT had reduced serum AP concentrations in the luteal phase (when symptoms of PMT are apparent) of the menstrual cycle in comparison with healthy controls. Seizures that occur in catamenial epilepsy are coupled with the changing levels of production of AP from progesterone [366, 441]. Even the drugs given to treat such disorders, such as tricyclics and selective serotonin reuptake inhibitors, have an effect on AP secretion [467]. Also, finasteride, when taken for the treatment of androgentic alopecia in human males, induces depressive like symptoms [468].

AP therefore appears to have important anxiolytic actions around the peri-partum period in both humans and rodents. New research therefore should focus on how manipulations of AP levels affect anxiety and how this impacts upon maternal behaviour. There are several possible lines of research to follow. One is to examine the effects of finasteride administration during lactation on the pup retrieval task in a

novel (i.e. stressful) cage. One would hypothesise that finasteride, by blocking AP production, would increase anxiety and hence impede the lactating rat in performing the pup retrieval task. Another line of experimentation would be to investigate the actions of AP in the high anxiety behaviour (HAB) line of rats. HAB rats are highly anxious and express excessive aggression towards conspecifics during a maternal aggression task [320]. AP administration to HAB rats during lactation would be proposed to reduce anxiety to normal levels seen in a lactating rat and hence reduce the aggressive behaviour displayed during a maternal aggression test. Other experiments could examine what would be the actions of AP in virgin female rats on anxiety behaviour expression and determine how this would then affect the pup-sensitization process. Administration of AP has already been seen to reduce anxiety in virgin female rats to levels of pregnant rats on the EPM, therefore one would hypothesise that this reduction in anxiety following injection of AP would help them overcome their aversion to pups and thus decrease latency to display full maternal behaviour in a pup-sensitization task [469].

In this study evidence suggests that, although AP may not have a direct influence on maternal behaviour, AP could play an important role in reducing fear and anxiety behaviour during the peri-partum period to allow for the expression of maternal aggression and other important maternal behaviours to ensure offspring survival.

Chapter Six: Maternal aggression and GABA

6.1 Introduction

In the previous chapter, the effects of AP on maternal behaviour including maternal aggression were investigated. It was discovered that because of the ability of AP to potentiate the anxiolytic actions of $GABA_A$ receptor functioning, AP may indirectly impact upon maternal aggression [356-359, 361]. If AP is able to modulate maternal aggression through enhancement of $GABA_A$ receptor functioning then GABA neurotransmission itself may control maternal aggression. This study therefore investigates the effect of direct manipulation of GABA neurotransmission on maternal behaviour, including maternal aggression.

For male rats, the relationship between GABA and aggression is well established [342, 373, 378, 380]. Depaulis *et al* (1983) reported that an ICV injection of the GABA antagonist, bicuculline methiodide (BM), decreased the aggressive actions of killer rats in the mouse-killing behaviour (MKB) paradigm without affecting the expression of other behaviours [470]. Furthermore, administration of the GABA agonist, 4,5,6,7-tetrahydroisoxazolo-(5,4-c-)-pyrindin-5-ol (THIP), decreased attack and killing latencies in killer males and elicited killing behaviour in males rats that were previously determined to be non-killer [470]. It was observed that there were resemblances between MKB and offensive behaviour displayed in the resident intruder paradigm hence it was hypothesised that they may be facilitated by similar mechanisms in the brain [471]. As in MKB, ICV injection of THIP induced offensive male aggression to a conspecific in a neutral cage [471]. ICV BM treatment in this paradigm decreased aggression by reducing the number of attacks, offensive sideways and upright posture behaviours and increased defensive behaviour [471].

On the other hand, in the shock-induced fighting paradigm the converse occurred as the GABA antagonists, picrotoxin and BM, were found to increase the number of fights [471, 472]. A possible reason for this discrepancy in behaviour could be because the shock induced fighting paradigm is actually measuring a defensive reaction rather than offensive [471, 472]. GABAergic control is therefore irrefutably necessary for male aggression where GABA neurotransmission enhances offensive aggressive and reduces defensive behaviour. Few studies to date have examined in detail whether the same is true for maternal aggression.

GABAergic neurotransmission is known to have important functions around the peri-partum period. The disinhibition of $GABA_A$ receptors just prior to parturition allows the necessary increase in OXT secretion for parturition and lactation. It also plays a role in the control of blood volume during pregnancy through its mediation by AP [445]. A significant correlation between maternal behaviour and the activation of glutamate decarboxylase (GAD) synthesising neurons in the MPOA, ventral BnST and ventrocaudal PAG implicates GABA neurotransmission as having a regulatory role in the control of maternal behaviour [355]. The role of GABA in maternal behaviour, especially maternal aggression, is confusing and complex. In the control of maternal behaviour, it appears that if GABA neurotransmission is enhanced in the MPOA of the lactating rat through direct infusion of GABA agonists, musimicol or baclofen, maternal behaviour is disrupted [438]. The MPOA plays a central role in regulating many components of maternal behaviour as both electrical and excitotoxic MPOA lesions disrupt most if not all maternal behaviour [27, 80, 82, 83, 158, 438]. However, Lee and Gammie (2007) reported that systemic administration of a $GABA_A$ receptor agonist, chlorodiazepine, enhanced maternal aggression in mice [158]. This

disparity may be due to a species difference as rats were used in the former study. Yet, Hansen *et al* (1985) reported that following an i.p. administration of a benzodiazepine antagonist into a lactating rat, maternal aggression was significantly decreased [237]. Furthermore, the administration of BM directly into the ventromedial hypothalamus and amygdaloid complex significantly decreased aggressive behaviour especially attacks and lateral posture [390]. BM administration directly into the LS of lactating mice resulted in significantly less maternal aggression [387]. These results suggest that GABA may exert its influence on aggression in a similar way in both sexes but further research is warranted before this is concluded.

Taken together the research above provides evidence for a regulatory role of GABA. However the extents to which $GABA_A$ receptors are involved during the peri-partum period to regulate maternal behaviour, specifically maternal aggression, are still not defined. The aim of this chapter was to test the hypothesis that a GABA antagonist, specifically BM, would decrease the maternal aggression when given directly via ICV into the brain of a lactating rat. In addition to examining the BM effects on lactating rats, the effects of BM administration to virgin female rats sensitized to pups and displaying maternal behaviour were also investigated. Virgin female rats will only display maternal behaviour after exposure to pups and little is known about whether the same mechanisms which control maternal behaviour in lactating rats work. The aim therefore of this study is to examine if manipulating GABA neurotransmission is able in influence maternal aggression in virgin female rats in the same as in lactating rats. A final aim was investigate where GABA may be acting to control maternal aggression in the brain; this was done by performing

double ICC for GAD 65/67 and Fos on the brain of a lactating rat following a maternal aggression test.

6.2 The effect of the GABA antagonist, bicuculline methiodide, on maternal aggression in the lactating rat

6.2.1 Method

On lactation day 2, each rat was surgically implanted with an ICV guide cannula. The rats were then allowed to recover for 2 days in their home cage. On the day of behavioural testing, an internal cannula was inserted into the guide cannula and by means of a Hamilton syringe 5µl of sterile 0.9% physiological saline (Genus Xpress, n=9) or 100ng of BM (1mg in 50ml sterile 0.9% physiological saline; Sigma; n=14) was slowly infused over 1 min. Three min following ICV infusion, lactating rats were submitted to a 10 min maternal aggression test. Rats were perfused 90 min after the start of testing and the brains collected and processed for Fos ICC.

Fos expression was examined in the LS, BnST, MPOA, SON, MeA, CeA, PVN and PAG using the technique described in chapter 2.

6.2.1.1 Statistics

A T-test was carried out to compare values for the two treatment groups when the data was normally distributed. If data was not normally distributed, a Mann-Whitney Rank Sum test was performed instead. Results were deemed statistically significant if $p \leq 0.05$.

6.2.2 Results

6.2.2.1 Aggressive behaviour

BM treatment significantly reduced the total time spent exhibiting aggressive behaviours ($p=0.042$, $t_{-2.17}$; vehicle (V)=10.29±3.1 %Total Time (TT), BM=4.13±1.2 %TT; Fig. 6.1) including two specific aggressive behaviours, lunging ($p=0.026$, $T_{(8,14)}=125.00$; V=4.06±1.8 %Total Aggressive Time (TAT), BM=0.53±0.3 %TAT; Fig. 6.1) and rearing ($p=0.037$, $T_{(8,14)}=123.00$; V=3.68±1.7%TAT, BM=1.07±0.3%TAT; Fig. 6.1) compared to vehicle treatment. However, BM administration did not significantly reduce the number of attacks ($p=0.175$, $T_{(9,14)}=130.00$; V=5.50±1.7attacks, BM=2.43±0.6attacks; Fig. 6.2) or increase latency to attack ($p=0.95$, $t_{-0.06}$; V=167.30±36.9secs, BM=169.88±23.1secs; Fig. 6.2) compared to the vehicle treated group. No statistical significance was observed between the two treatment groups in the duration of any other aggressive behaviour (attack $p=0.23$, $T_{(8,14)}=110.00$, biting $p=0.23$, $T_{(8,14)}=110.00$, clawing $p=0.41$, $T_{(8,14)}=104.50$, and pinning down $p=0.21$, $T_{(8,14)}=111.00$; Fig. 6.3). There was no significant difference between the two treatment groups in sniffing behaviour ($p=0.052$, $t_{1.88}$) however there was a trend for the BM treated group to sniff more.

6.2.2.2 Maternal behaviour

There was no significant difference in the expression of maternal behaviour either as a percentage of total time ($p=0.12$, $T_{(8,14)}=115.00$; Fig. 6.1) or when broken down into individual behaviours and compared as percentage of total maternal time (pup moving $p=0.43$, $T_{(9,14)}=95.00$, general pup $p=0.054$, $T_{(9,14)}=139.00$, nesting $p=0.63$, $T_{(9,14)}=116.00$ or nursing $p=0.80$, $T_{(9,14)}=103.50$).

6.2.2.3 General behaviour

The BM treated group spent significantly more time expressing general behaviours compared to the vehicle treated group ($p=0.027$, $T_{(8,14)}=59.00$; V=40.59±3.1%TT, BM=61.19±5.7%TT; Fig. 6.1). The vehicle treated group spent more time self grooming compared to the BM treated group ($p=0.032$ $T_{(8,14)}=124.00$; V=24.56±4.8% total general time (TGT), BM=11.54±2.2%TGT; Fig. 6.4).

6.2.2.4 Response to aggression behaviour by the resident

Treatment with BM did not result in a significant change in the response to aggressive behaviour from the intruder by the resident rat compared to the vehicle treatment ($p=0.66$, $T_{(8,14)}=85.00$, V=0.42±0.2%TT, BM=1.94±1.0%TT; Fig. 6.1).

6.2.2.5 Fos expression

BM treatment had no significant effect in the number of Fos positive cells quantified in any specific brain region examined (BnST $p=0.64$ $T_{(9,14)}=100.00$, LS $p=0.78$, $T_{(9,14)}=113.00$, SON $p=0.16$, $T_{(9,13)}=125.00$, MPOA $p=0.47$, $T_{(9,14)}=120.00$, CeA $p=0.37$, $t_{0.92}$, MeA $p=0.64$, $T_{(9,14)}=116.00$, parvocellular PVN $p=0.21$, $t_{-1.30}$, magnocellular PVN $p=0.49$, $T_{(9,14)}=119.50$ or PAG $p=0.86$, $t_{0.18}$; Fig. 6.5) compared to the vehicle treatment.

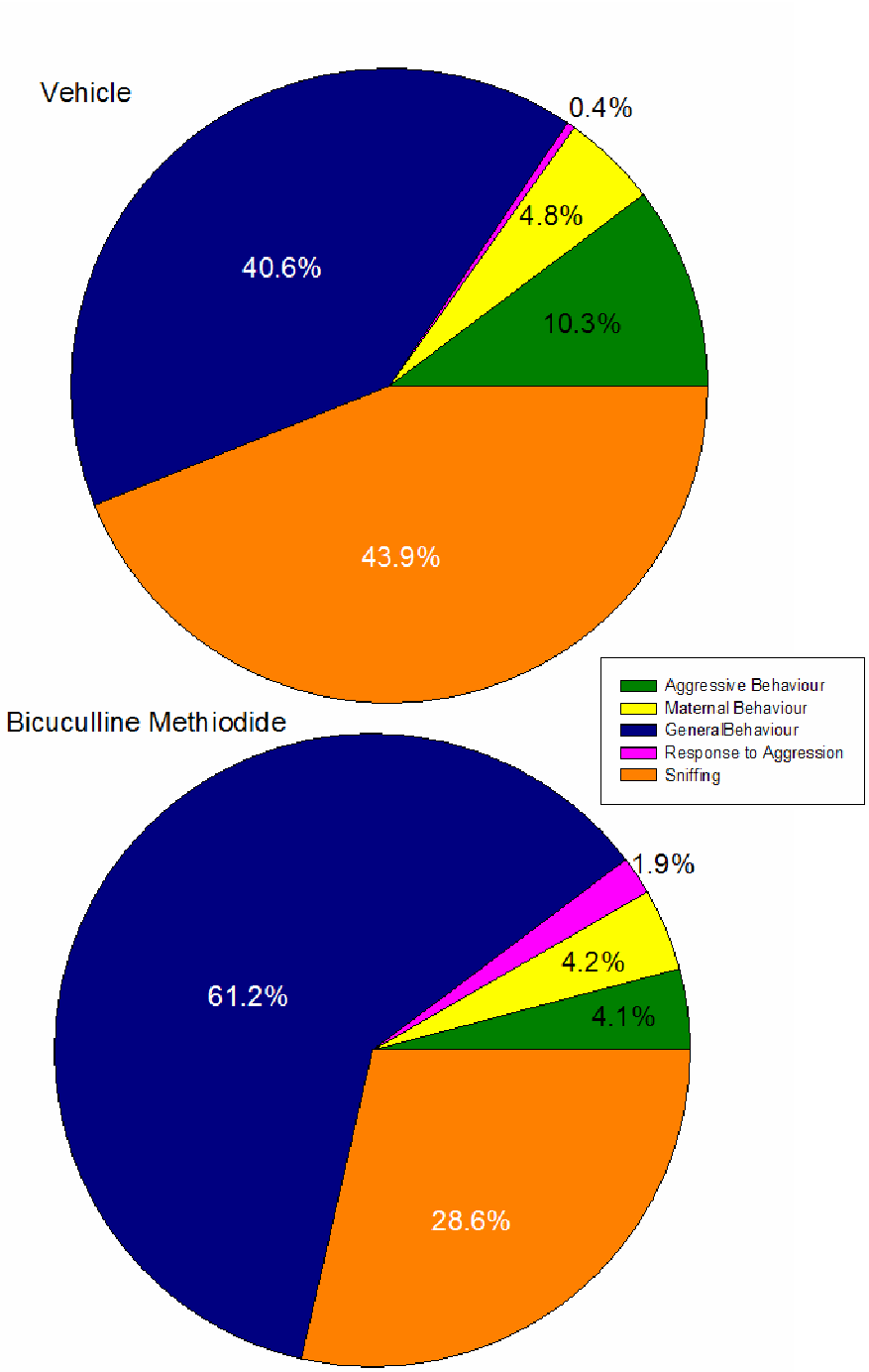
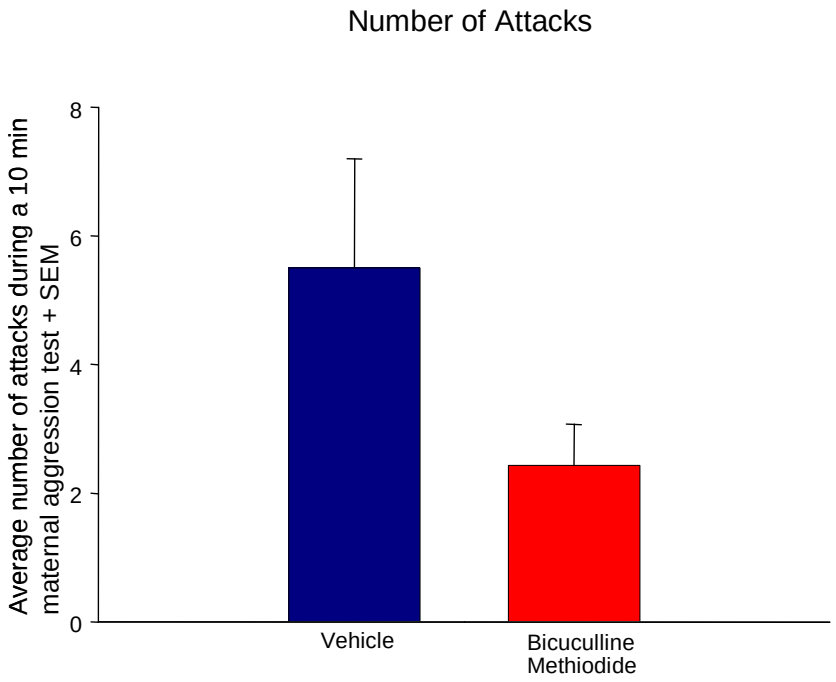


Figure 6.1: Average percentage of total time spent exhibiting different behaviours by the resident lactating rat during a maternal aggression test following ICV administration of bicuculline methiodide. The bicuculline methiodide (BM) treated group (n=14) were infused with 100ng of BM in physiological saline into the lateral ventricle 3 min prior to testing. The vehicle treated group (n=9) were infused with physiological saline at the same time point. Pie charts depicting the mean percentage of total time of the maternal aggression test (10 min) spent exhibiting aggressive, maternal, response to aggression or general behaviours by the resident lactating rat in her home cage with pups present. Note the significant decrease in percentage of total time spent expressing aggressive behaviours and significant increase in general behaviour exhibition in the BM treated group compared to the vehicle treated group.



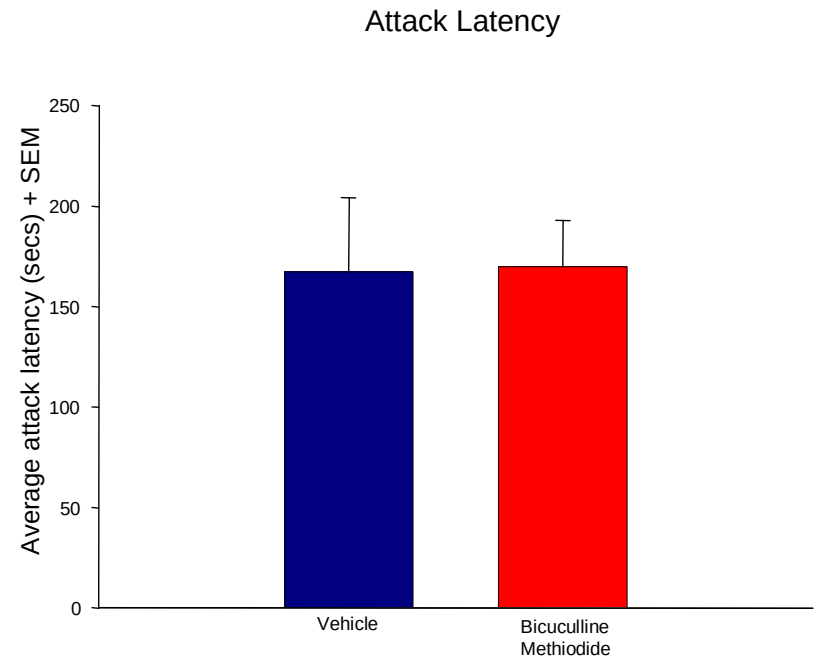


Figure 6.2: Average number of attacks and attack latency for the resident lactating rat during a maternal aggression test following ICV administration of bicuculline methiodide. The bicuculline methiodide (BM) treated group (n=14) were infused with 100ng of BM in physiological saline into the lateral ventricle 3 min prior to testing. The vehicle treated group (n=9) were infused with physiological saline at the same time point. The average number of attacks and latency to attack (secs) the virgin intruder during a 10 min maternal aggression was compared between the BM and vehicle treated groups. Data are represented as mean + SEM.

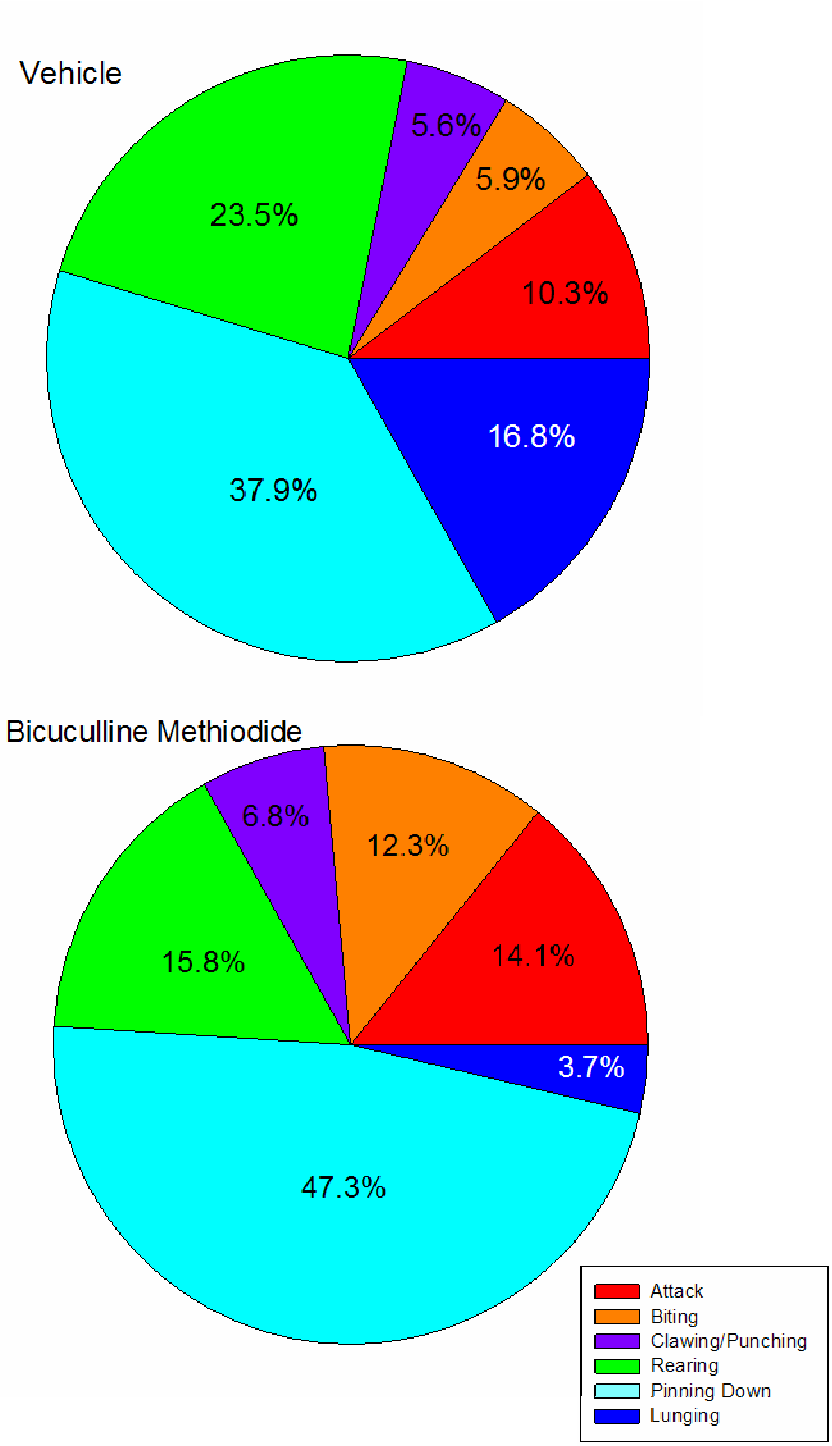


Figure 6.3: Average percentage of total aggressive time spent exhibiting different aggressive behaviours by a resident lactating rat during a maternal aggression test following ICV administration of bicuculline methiodide. The bicuculline methiodide (BM) treated group (n=14) were infused with 100ng of BM in physiological saline into the lateral ventricle 3 min prior to testing. The vehicle treated group (n=9) were infused with physiological saline at the same time point. The average percentage of total aggression time by the resident spent exhibiting attacking, biting, clawing/punching, rearing, pinning down, lunging or sniffing behaviours towards a novel female intruder during a 10 min maternal aggression test with the pups present. There was no significant difference in the expression of any specific aggressive behaviour between the two treatment groups.

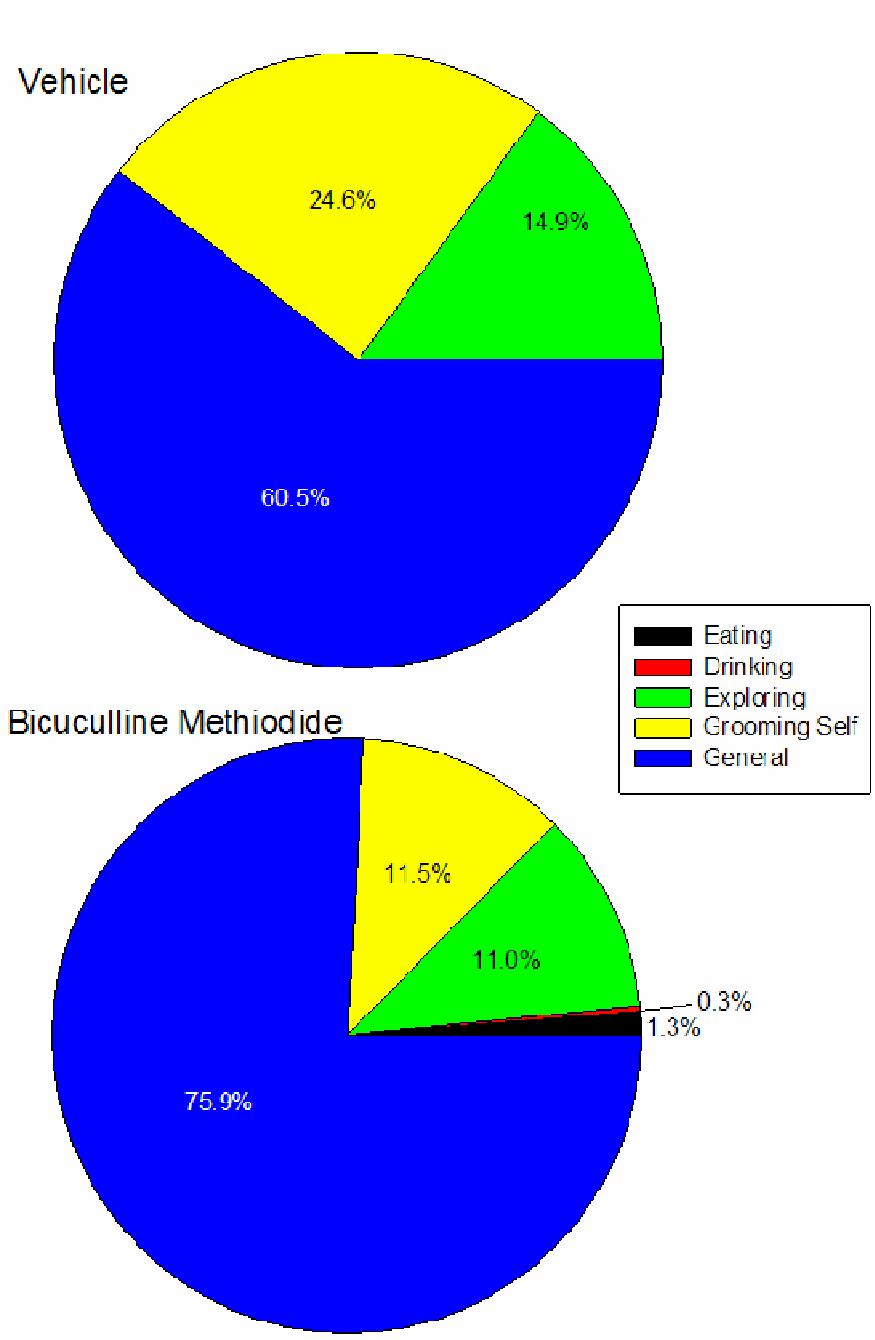


Figure 6.4: Average percentage of total general time spent exhibiting different general behaviours by a resident lactating rat during a maternal aggression test following ICV administration of bicuculline methiodide. The bicuculline methiodide (BM) treated group (n=14) were infused with 100ng of BM in physiological saline into the lateral ventricle 3 min prior to testing. The vehicle treated group (n=9) were infused with physiological saline at the same time point. The average percentage of total general time spent by the resident eating, drinking, exploring, grooming self or general (defined as moving around cage with no involvement of intruder or pups) behaviour during a 10 min maternal aggression test with the pups present. Note that the BM treated group spent significantly less time self grooming than the vehicle treated group.

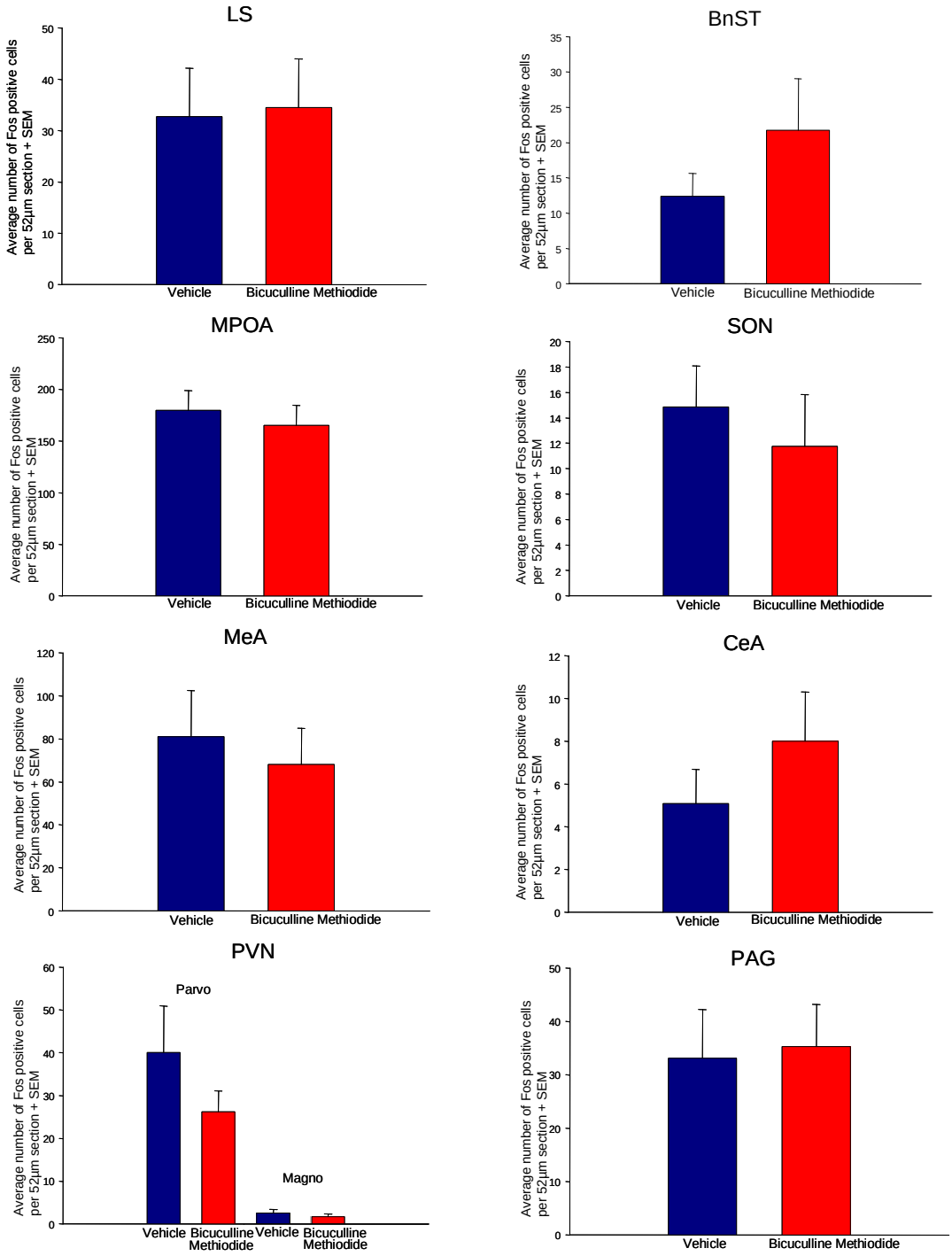


Figure 6.5: Average number of Fos positive cells in specific brain areas of the lactating rat brain following a maternal aggression test and ICV administration of bicuculline methiodide. Lactating rats were infused with 5µl of bicuculline methiodide (1mg dissolved in 50ml sterile physiological saline) or physiological saline by means of a Hamilton syringe through an internal cannula into the lateral ventricle 3 min prior to maternal aggression test. Expression of Fos in the lateral septum (LS), bed nucleus of stria terminalis (BnST), medial preoptic area (MPOA), supraoptic nucleus (SON), medial and central amygdala (MeA and CeA respectively), parvocellular paraventricular nucleus (PVN) and periaqueductal grey area (PAG) of Bicuculline Methiodide (n=14) or vehicle (n=9) treated rats was quantified by performing Fos immunocytochemistry on brains perfused 90 min after the start of behavioural testing. Data are represented as mean + SEM.

6.3 The effect of a GABA antagonist, bicuculline methiodide, on maternal aggression in pup-sensitized virgin rats

6.3.1 Method

Virgin females were randomly assigned to one of three treatment groups; non-surgical control, vehicle or GABA Antagonist. Vehicle and GABA antagonist groups were implanted with an ICV guide cannula and left for 2 day recovery period. A non-surgical control group, with no ICV cannulation, was included to ensure that any behavioural or Fos differences between vehicle and BM treated groups were the result of treatment and not ICV cannulation. Following surgery or not, all rats underwent pup-sensitization with daily monitoring for the expression of maternal behaviour (for details please refer to chapter 2). The day after the virgin was first defined as 'fully maternal', the pup-sensitization test was repeated. Once completed, if the virgin was not cannulated i.e. the non-surgical control group, the rat was exposed to a 30 min maternal aggression test with donor pups (n=3) present in her home cage. If the virgin rat was cannulated, the GABA antagonist group received a 5µl infusion of 100ng of BM (1mg in sterile 50ml of physiological saline) whilst the vehicle group received 5µl of 0.9% physiological saline by means of a Hamilton syringe via an internal cannula 3 min prior to performing the maternal aggression test as uncannulated rats above. Immediately after the maternal aggression tests, a vaginal smear was taken from the pup-sensitized rat and analysed to see what stage of cycling she was at. Rats were perfused 90 min after the start of testing and the brains collected and processed for Fos ICC.

Fos expression was examined in the LS, BnST, MPOA, SON, MeA, CeA, PVN and PAG using the technique described in chapter 2.

6.3.1.1 Statistics

A one-way ANOVA was carried out to compare values for the three groups when the data was normally distributed. If data was not normally distributed, an ANOVA on Ranks was performed instead. Results were deemed statistically significant if $p \leq 0.05$.

6.3.2 Results

6.3.2.1 Pup-sensitization

Thirty virgin female rats began the pup-sensitization procedure. Of those 30, 7 were removed from the experiment because they killed the pups during the first day of testing. Another 2 were removed for other experimental reasons, one had the wrong cannula placement and the other became injured so had to be put down. Of the remaining 21, 15 became maternal and 6 never became maternal even after 13 days of pup exposure. The overall average length for virgin female rat to become maternal was 7.89±0.8days, there was no significant difference between the treatment groups in latency to become maternal ($p=0.46$, $F_{(2,17)}=0.81$). All virgin female rats that performed the maternal aggression test after becoming maternal were either in the proestrous or estrous stage of their cycle.

6.3.2.2 Aggressive behaviour

Overall, pup-sensitized virgin rats expressed little aggressive behaviour. In the whole experiment, only two pup-sensitized virgin rats attacked the intruder. Although both of these were in the GABA antagonist group, they only did this once during the maternal aggression test and their latency to attack was substantially higher than observed in normal lactating rats (pup-sensitized virgins=1541.00±106.00, vehicle group of experiment 6.2=167.30±36.9secs; $p<0.001$, $t_{-15.6}$). Thus, there was no

significant difference in display attack behaviour between the treatment groups (number of attacks p=0.10, H_2=4.59).

All pup-sensitized virgins displayed other forms of aggressive behaviour, including biting, clawing, lunging, rearing, pinning down but there was no difference between the treatment groups in total time exhibiting these behaviours (p=0.33, H_2=2.22; non-surgical control=12.12±4.1%TT, vehicle=28.34±11.1%TT, BM=7.46±2.7%TT; Fig. 6.6).

6.3.2.3 Maternal behaviour

Maternal behaviour was quantified to ensure that virgin rats were still displaying maternal care towards the pup during the maternal aggression test. All virgin pup-sensitized rats displayed some of the specific maternal behaviours during the maternal aggression test but there was no statistical difference between the treatment groups in the total time spent expressing all maternal behaviours (p=0.25, $F_{(2,17)}$=1.55; Fig. 6.6) or nesting alone (p=0.20, $F_{(2,17)}$=1.84; Fig. 6.7) therefore there is no effect of BM treatment or surgery on the ability to perform maternal behaviour.

6.3.2.4 General behaviour

BM treatment did not significantly alter general behaviour when examined as a percentage of total time compared to the vehicle treated and non-surgical control groups (p=0.055, $F_{(2,18)}$=3.49; Fig. 6.6). There was no significant effect of surgery on general behaviour expression either (p=0.055, $F_{(2,18)}$=3.49; Fig. 6.6).

6.3.2.5 Response to aggression behaviour by the resident

The percentage of total time displaying a behavioural response to aggression behaviour from the intruder was significantly higher following BM treatment compared with the non-surgical control group (p=0.019, H_2=7.90; Fig. 6.6) but not

the vehicle treated group ($p>0.05$, $H_2=7.90$; Fig. 6.6). There was no significant effect of surgery when the non-surgical control group was compared to the vehicle treated group ($p>0.05$, $H_2=7.90$; Fig. 6.6). The duration of freezing behaviour in BM treated virgins was also statistically higher when compared to the non-surgical control group ($p=0.014$, $H_2=8.48$; Fig. 6.6) but not to the vehicle treated group ($p>0.05$, $H_2=8.48$; Fig. 6.6).

6.3.2.6 Fos expression

Fos expression following maternal aggression was quantified in the same specific brain areas as those investigated for lactating rats following BM administration. In the BnST, Fos expression in BM treated pup-sensitized virgin rats was significantly increased in comparison to both the non-surgical control and vehicle treated groups ($p=0.029$, $F_{(2,16)}=4.61$; Fig. 6.8 and 6.9). Fos expression did not differ between the treatment groups in any other area (LS $p=0.16$, $H_2=3.64$; SON $p=0.84$, $H_2=0.35$; MPOA $p=0.99$, $F_{(2,17)}=0.01$; MeA $p=0.90$, $F_{(2,18)}=0.12$; CeA $p=0.088$, $H_2=4.86$; PVN $p=0.052$, $H_2=5.92$ or PAG $p=0.20$, $F_{(2,16)}=1.79$; Fig. 6.8).

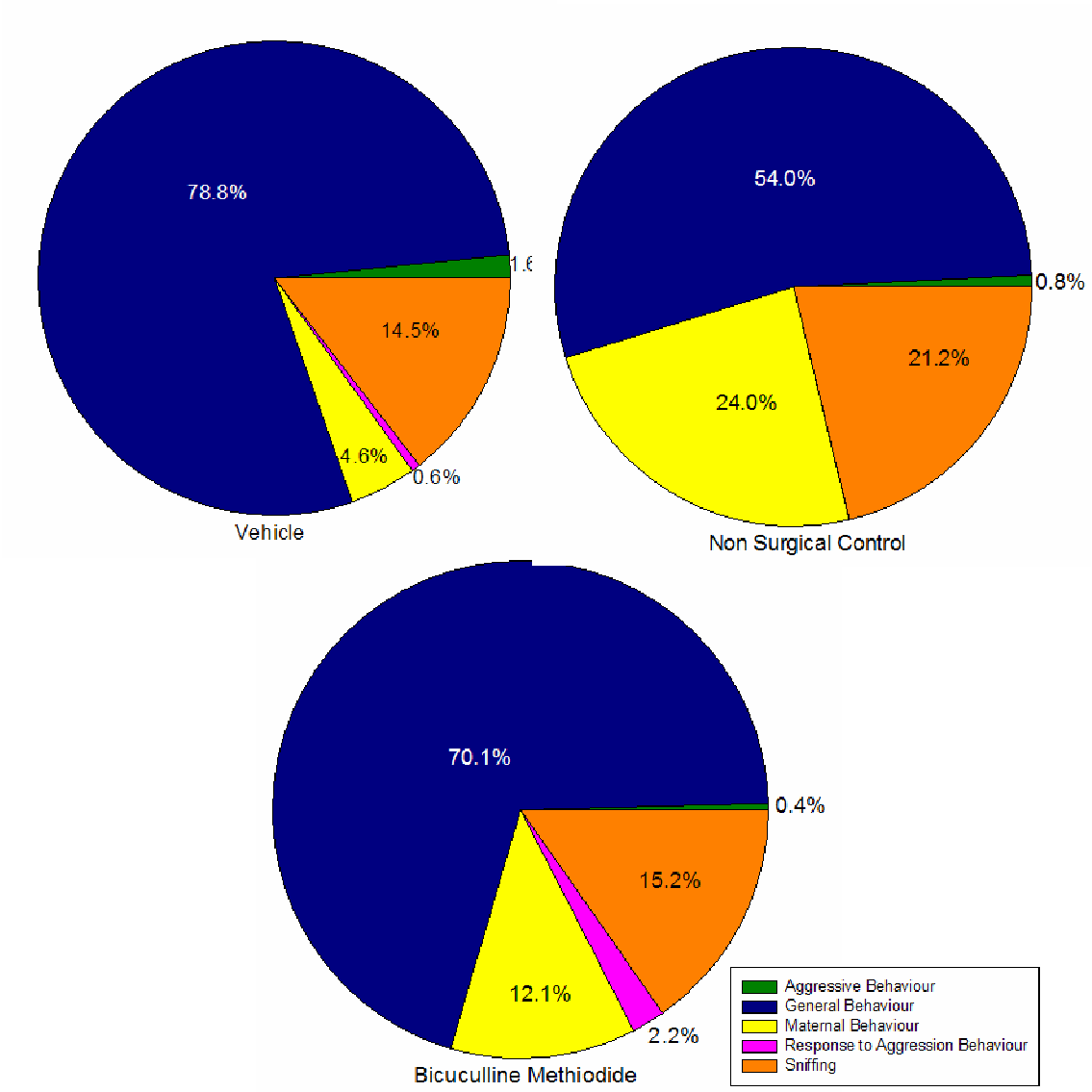


Figure 6.6: Average percentage of total time spent exhibiting different behaviours by the resident pup-sensitized virgin female rat following ICV administration of bicuculline methiodide. Pup-sensitized virgin female rats that were confirmed as fully maternal were injected with 5µl of bicuculline methiodide (BM: 1mg dissolved in 50ml sterile physiological saline) or vehicle (physiological saline) by means of a Hamilton syringe through an internal cannula into the lateral ventricle 3 min prior to a 30 min maternal aggression test. The non-surgical control group were left untreated prior to being subjected to a 30 min maternal aggression test. Pie charts depicting the mean percentage of total time of the maternal aggression test spent exhibiting aggressive, maternal, response to aggression or general behaviours by the pup-sensitized resident virgin female rat (bicuculline methiodide n=5, vehicle n=5, non-surgical control n=8) in her home cage with her pups present. One should note the significantly higher percentage of total time the BM treated group spent exhibiting response to aggression behaviour compared to the non-surgical control group.

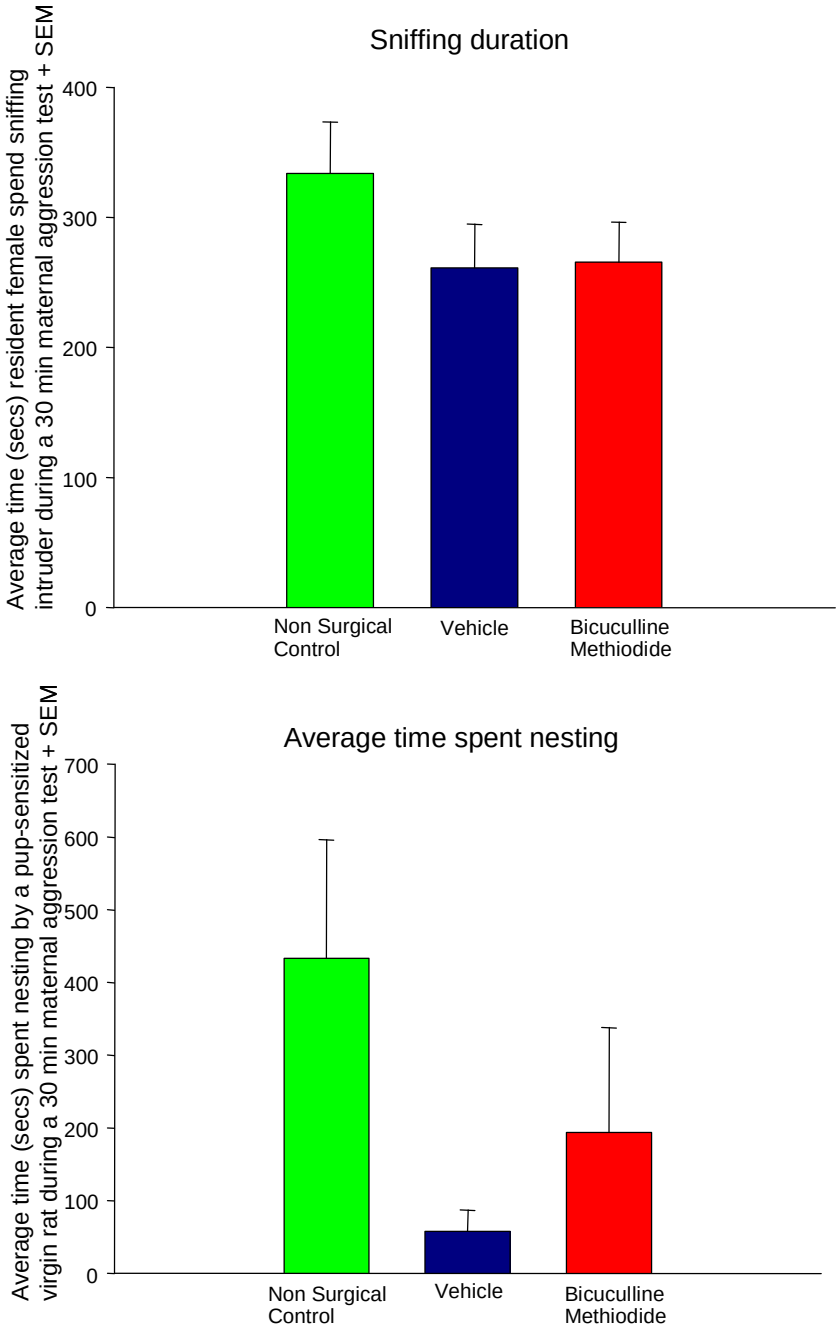


Figure 6.7: Average time spent sniffing or nesting during a maternal aggression test by the resident pup-sensitized virgin female rat following administration of bicuculline methiodide. Virgin female rats one day after being confirmed as fully maternal following sensitization to donor pups in their home cage and that were cannulated were injected with 5µl of bicuculline methiodide (1mg dissolved in 50ml sterile physiological saline) or vehicle (physiological saline) by means of a Hamilton syringe through an internal cannula into the lateral ventricle 3 min prior to a 30 min maternal aggression test. The non-surgical control group were left untreated prior to being subjected to a 30 min maternal aggression test. Average time in seconds spent sniffing the novel virgin female intruder or nesting on donor pups by the pup-sensitized resident virgin female rat (Biucuclline Methiodide n=5, vehicle n=5, non-surgical control n=8) in her home cage during a maternal aggression test. Data are represented as mean + SEM.

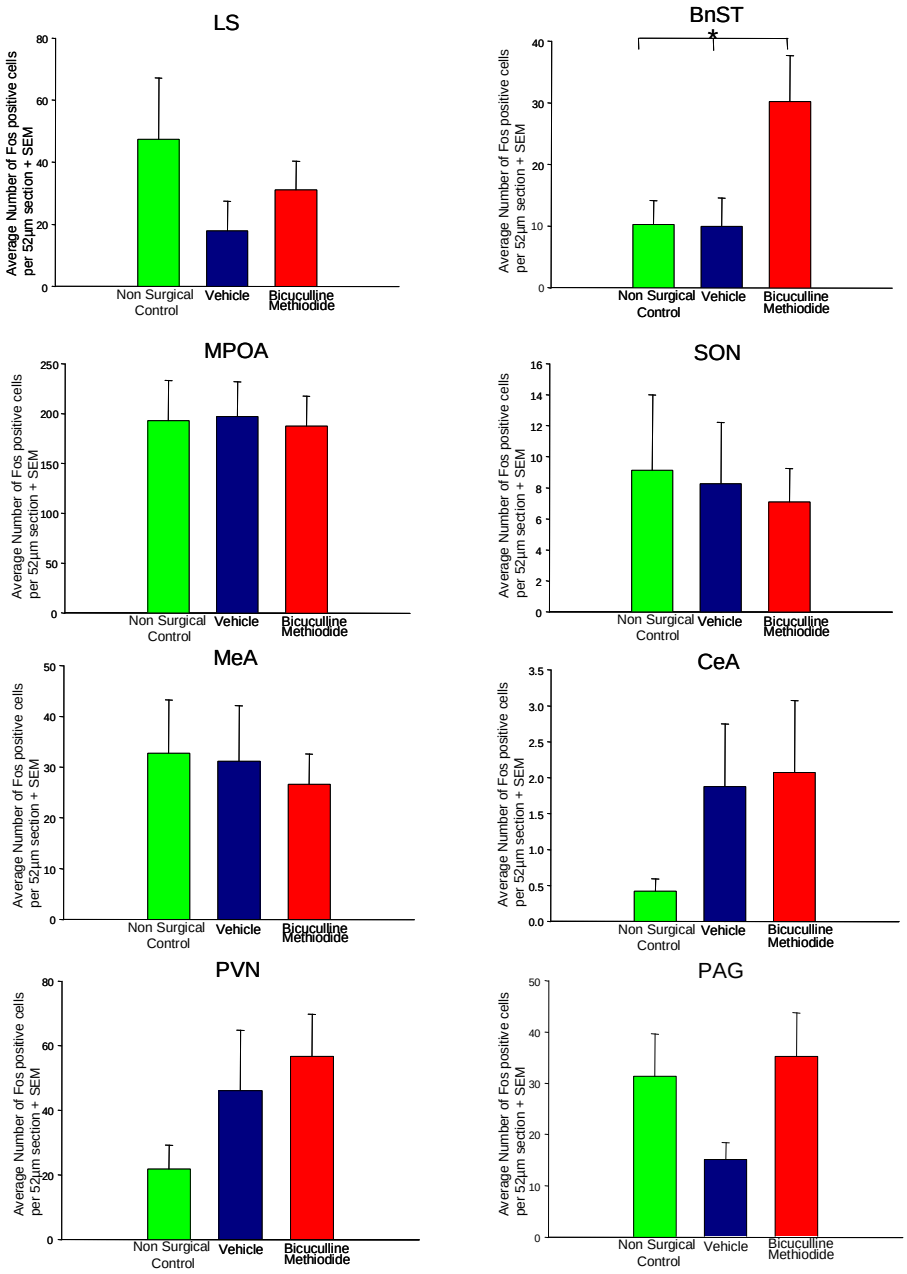


Figure 6.8: Average number of Fos positive cells in specific brain areas of the resident pup-sensitized virgin rat following a maternal aggression test and ICV administration of bicuculline methiodide. Virgin female rats were sensitized to 3 donor pups in their home cage until they displayed full maternal behaviour. One day after being confirmed as fully maternal, cannulated pup-sensitized virgin female rats were injected with 5µl of bicuculline methiodide (1mg dissolved in 50ml sterile physiological saline) or vehicle (physiological saline) by means of a Hamilton syringe through an internal cannula into the lateral ventricle 3 min prior to a 30 min maternal aggression test. The non-surgical control group were left untreated prior to being subjected to a 30 min maternal aggression test. Expression of Fos in the lateral septum (LS), bed nucleus of stria terminalis (BnST), medial preoptic area (MPOA), supraoptic nucleus (SON), medial and central amygdala (MeA and CeA respectively), parvocellular paraventricular nucleus (PVN) and periaqueductal grey area (PAG) of Bicuculline Methiodide (n=5) or vehicle (n=5) treated or non-surgical control (n=8) groups was quantified using Fos immunocytochemistry on brains perfused 90 min after behavioural testing. Data are represented as mean ± SEM. *=p≤0.05

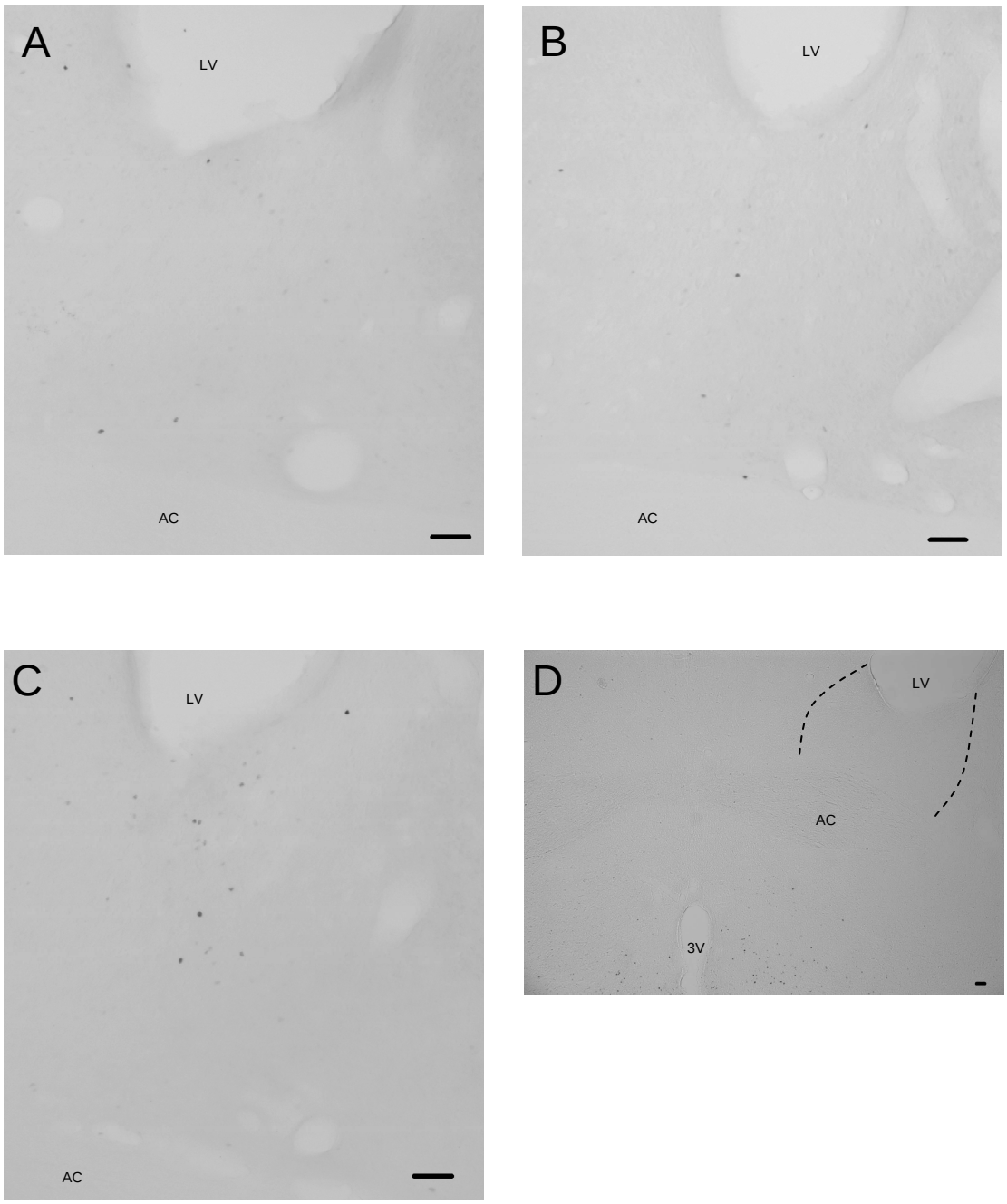


Figure 6.9: Photographs of Fos positive cells in bed nucleus of stria terminalis pup-sensitized female virgin rats after a maternal aggression test and ICV administration of bicuculline methiodide. Pup**-s**ensitized virgin female rats that were confirmed as fully maternal were injected with 5µl of bicuculline methiodide (1mg dissolved in 50ml sterile physiological saline) or vehicle (physiological saline) by means of a Hamilton syringe through an internal cannula into the lateral ventricle 3 min prior to a 30 min maternal aggression test. The non-surgical control group were left untreated prior to being subjected to a 30 min maternal aggression test. Photographs depicting Fos positive cells in the BnST in the non surgical control (n=8, A), vehicle (n=5, B) or bicuculline methiodide (n=5, C) treated pup-sensitized female virgin rats following a 30 min maternal aggression test. D shows a lower power photograph of the BnST area (marked with dashed lines). Scale bars = 50µm. Abbreviation: AC= anterior commisure, LV= lateral ventricle, 3V = third ventricle.

6.4 Mapping GAD containing cells in the maternal aggression circuitry in the lactating rat

6.4.1 Method

Brains were collected from lactating rats perfused 90 min that were subjected to a 30 min maternal aggression test (AT; n=9) or left undisturbed (NAT; n=8) with their pup present. Fos and GAD65/67 double ICC was performed as described in the Chapter 2 and examined in the LS, BnST, MPOA, SON, MeA, CeA, PVN and PAG (brain regions already linked to maternal aggression). For rats exposed to a maternal aggression test, aggressive behaviour was analysed to ensure it was expressed at levels previously recorded.

6.4.1.1 Statistics

A T-test was performed to compare values for the two groups when the data was normally distributed. If data was not normally distributed, a Mann-Whitney Rank Sum test was performed instead. Results were deemed statistically significant if $p \leq 0.05$.

6.4.2 Results

6.4.2.1 Aggressive behaviour

Aggressive behaviour in aggression tested rats was comparable to levels observed in vehicle treated lactating rats from the AP and maternal behaviour study (chapter 4). The average latency to attack was 194.0±63.6 secs (vehicle group for AP and maternal aggression experiment was 167.1±38.8; p=0.86, $T_{(9,9)}$=88.0) and average number of attacks was 10.0±2.3 (vehicle group for AP and maternal aggression experiment was 8.2±1.7; p=0.57, $T_{(9,9)}$=92.5).

6.4.2.2 GAD65/67 and Fos positive cells expression

There were significantly fewer GAD65/67 cells activated in the SON ($p=0.013$, $t_{2.92}$; NAT=18.77±1.6, AT=13.50±1.0; Fig 6.10 and 6.11) but significantly more in the MeA ($p<0.001$, $T_{(7,8)}=41.00$; NAT=11.57±0.9, AT=25.12±2.1; Fig. 6.10 and 6.11) following an aggression test.

There was no significant difference in the number of cells double labelled for GAD65/67 and Fos observed in the BnST ($p=0.24$, $t_{1.23}$), LS ($p=0.84$, $t_{0.21}$), MPOA ($p=0.53$, $t_{-0.65}$), CeA ($p=0.21$, $t_{-1.32}$), Parvocellular ($p=0.46$, $t_{(7,8)}=49.00$) or Magnocellular ($p=0.88$, $t_{(7,8)}=58.00$) PVN or PAG ($p=0.55$, $t_{-0.62}$; Fig. 6.10).

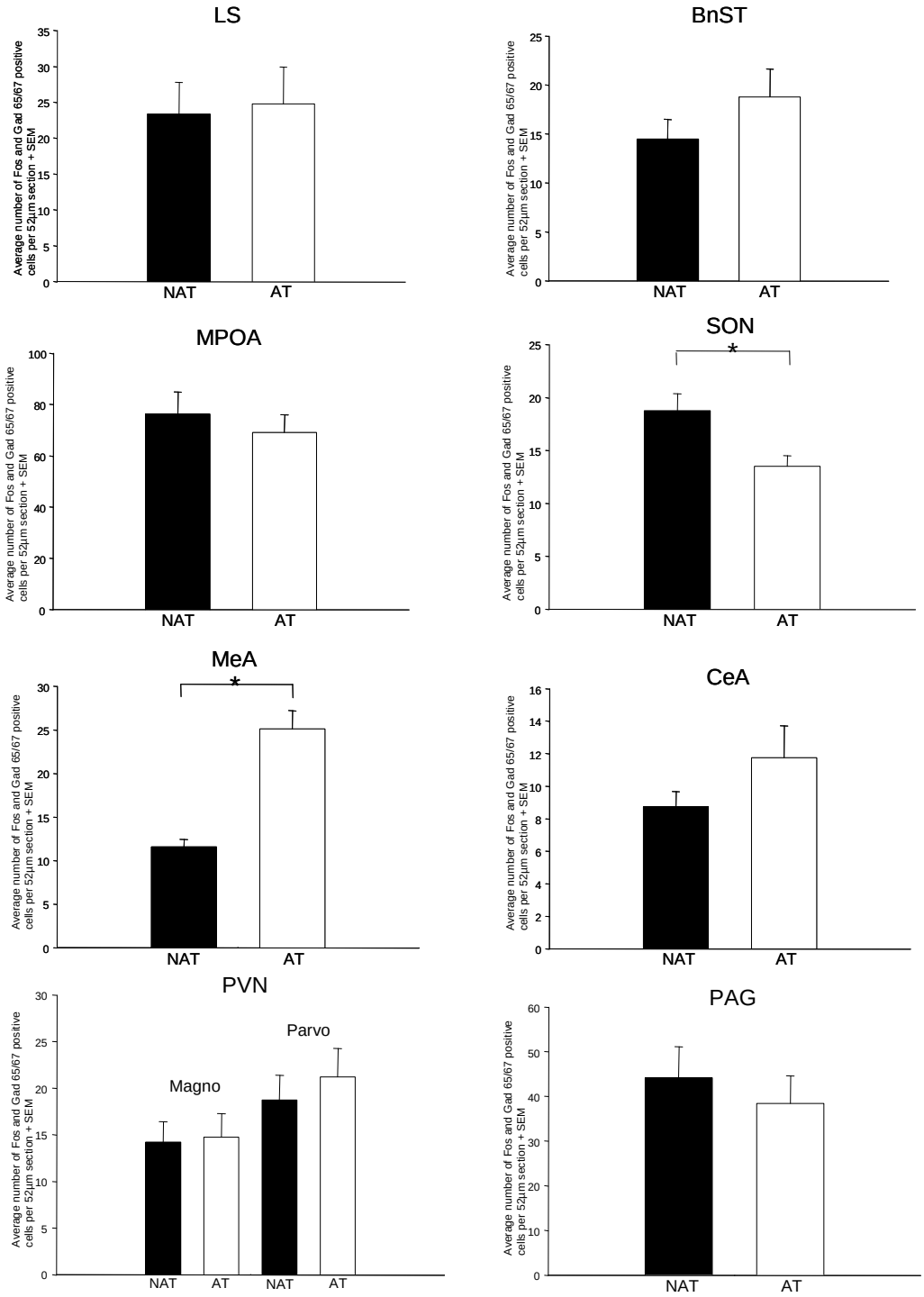


Figure 6.10: Average number of Fos and GAD 65/67 positive cells in specific brain areas of the resident lactating rat following a maternal aggression test. Lactating rats were subjected to a 30 min maternal aggression test or left in their home cage undisturbed with their pup present (n=8-18). Expression of Fos and GAD65/67 cells the lateral septum (LS), bed nucleus of stria terminalis (BnST), medial preoptic area (MPOA), supraoptic nucleus (SON), medial and central amygdala (MeA and CeA respectively), parvocellular (parvo) and magnocellular (magno) paraventricular nucleus (PVN) and periaqueductal grey area (PAG) of aggression tested (AT, n=9) and non aggression tested (NAT, n=8) rats was examined using double immunocytochemistry on brains perfused 90 min after behavioural testing. Data are represented as mean ± SEM. *=p<0.0

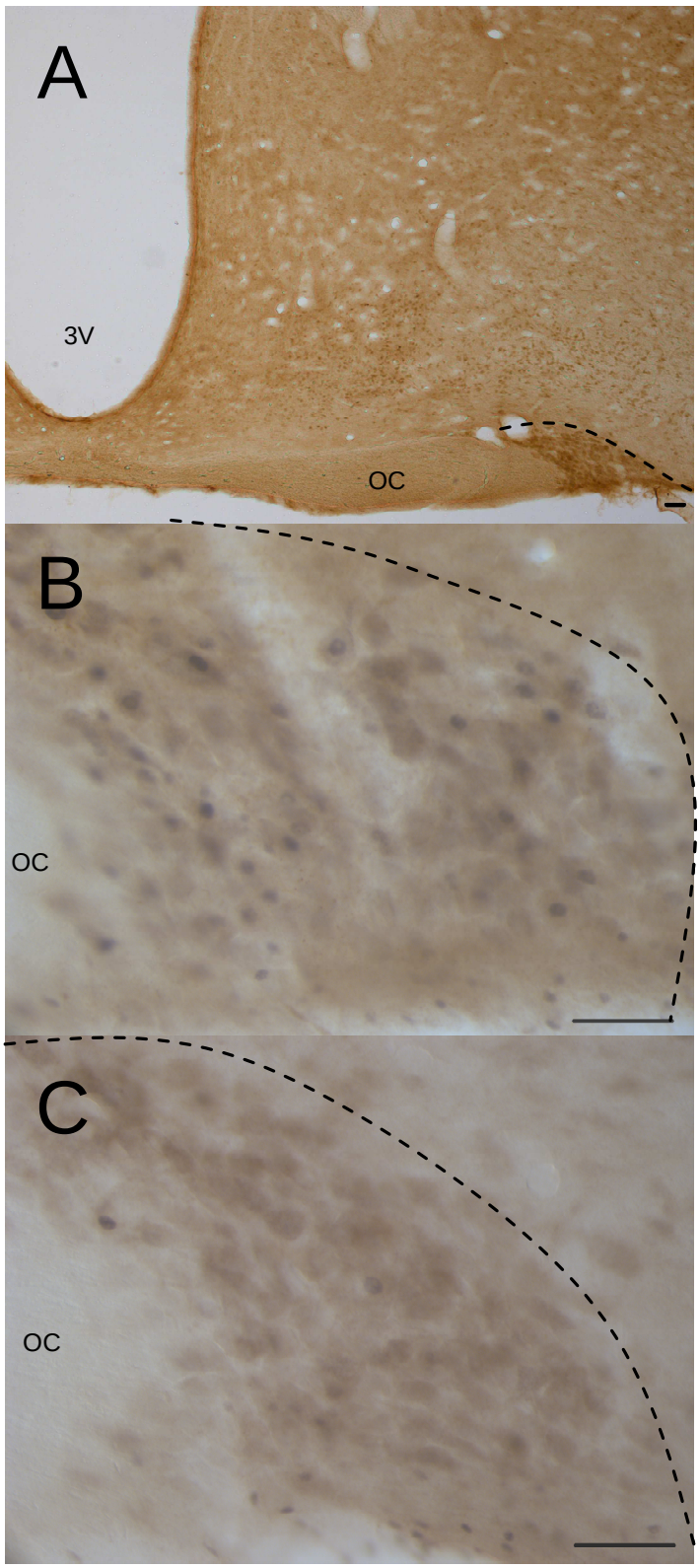


Figure 6.11: Photographs of double labelled GAD65/67 and Fos positive cells in the supraoptic nucleus of lactating rats following a maternal aggression test. Photographs depicting Fos positive cells in the supraoptic nucleus (defined by black dashed line, as shown in A) in the lactating rats (non aggression tested, n=8, and aggression tested, n=9; B and C respectively). Scale bars = 50μm. Abbreviation: 3V = third ventricle, OC= optic chiasm.

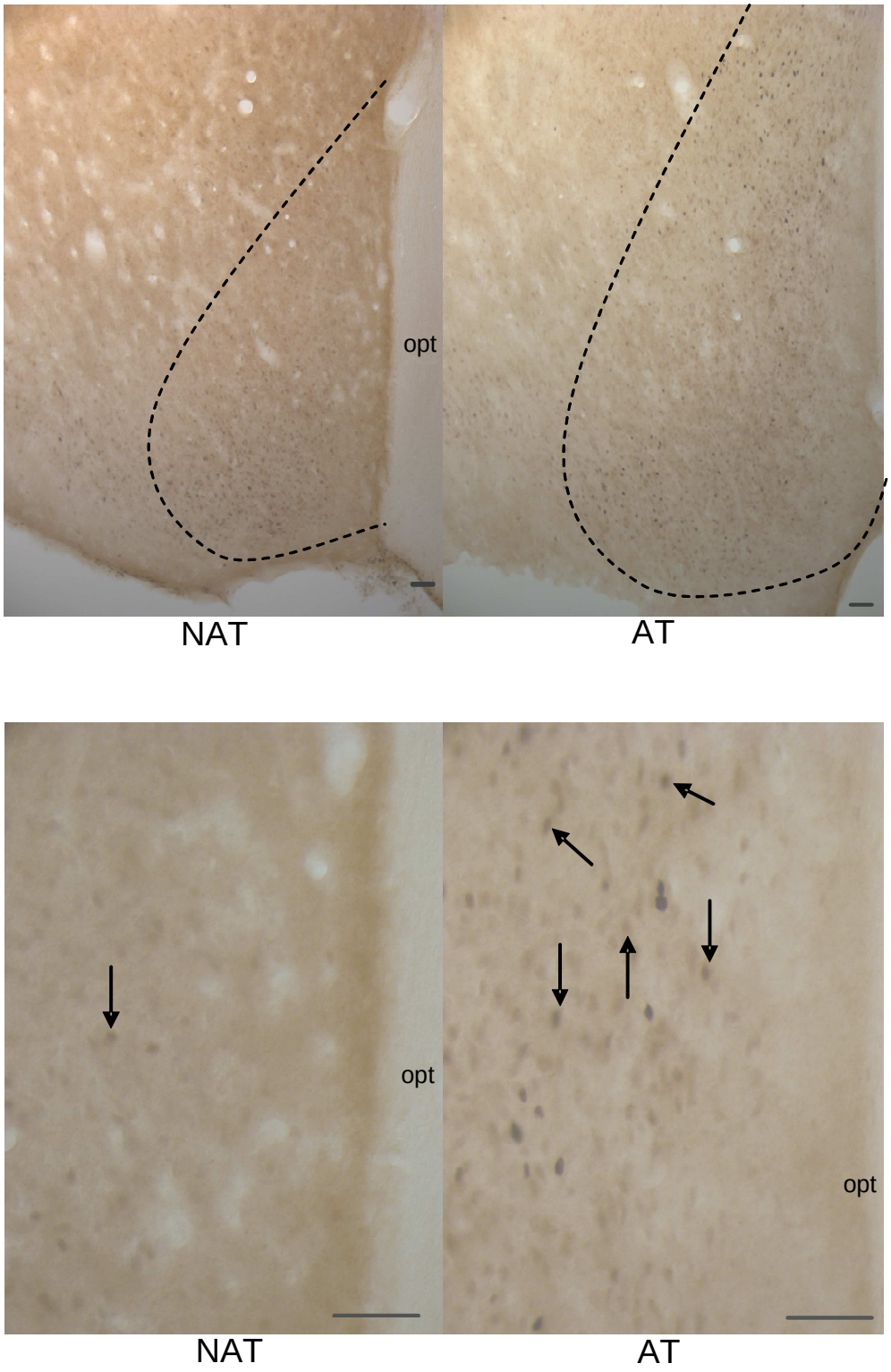


Figure 6.12: Photographs of double labelled GAD65/67 and Fos positive cells in the medial amygdala of lactating rats following a maternal aggression test. Photographs depicting Fos and GAD65/67 positive cells (black arrows) in the medial amygdala (defined by black dashed line) in the lactating rats (non aggression tested, NAT, n=8, and aggression tested, AT, n=9) at low (A and B) and high (C and D) power. Scale bars = 50μm. Abbreviation: opt=optic tract.

6.5 Discussion

Blocking GABA neurotransmission using the antagonist BM did not decrease number of attacks or latency to attack, but did significantly decrease total time spent displaying aggressive behaviour in lactating rats. BM treatment decreased the duration of lunging and rearing behaviours in particular. This is in accordance with Hansen and Ferreria (1986) who reported a significant decrease in frequency of lateral posture behaviour in lactating rats following direct injection of BM into the ventromedial hypothalamus and amygdaloid complex [390]. However in their study they do not mention upright or rearing behaviour so it is unknown whether they observed the same difference as the present study did [390]. In male aggression, Depaulis *et al* (1985) reported a decrease in duration in what they termed offensive sideways and upright posture behaviours, probably similar to the effects in the present study [471].

Similarities between changes in general behaviour in the BM treated lactating rat in the present study and the BM treated male can also be observed. A significant decrease in self grooming behaviour was found in BM treated lactating rats. This is in agreement with the results reported for male rats following administration of BM where cage exploration was increased and allogrooming was decreased [471]. Thus, the results of this study support the hypothesis that GABA neurotransmission enhances the expression of certain offensive aggressive behaviours.

It was mentioned in the introduction that GABA agonists, muscimol and baclofen, reduced maternal aggression, however this was only significant when examining percentage level of aggression [438]. There was no difference in number of attacks or latency to attack [438]. There is no analysis of individual aggressive

behaviours discussed, i.e. offensive (such as lunging, biting) or defensive (such as escape), so it is unknown in what specific aspect of maternal aggression this decrease occurred. Hence it may have been in defensive behaviours, which would be in keeping with the current theoretical idea that GABA enhances offensive behaviours whilst decreasing defensive ones. This form of control is not restricted to the rat. In the cat, direct administration of the GABA agonist, muscimol, into the PAG decreased the affective defence response but had no effect on the active biting predatory attack (offensive) responses [473].

During pregnancy and parturition, there are dramatic changes in the $GABA_A$ receptor complex that impact upon its functioning. During pregnancy there are significant decreases in the amount of mRNA of $GABA_A$ receptor subunits γ2l and α5 in the cerebral cortex, however at term these return to normal levels [443]. When the γ subunit is present in the $GABA_A$ receptor complex, an increase in the $GABA_A$ receptor sensitivity to benzodiazepines has been found. If both γ and α5 subunit included in the $GABA_A$ receptor complex, it expresses its highest affinity for GABA. Together these findings suggest that during pregnancy $GABA_A$ receptor functioning is decreased to protect from overstimulation by ovarian hormones and neurosteroids whose levels increase dramatically through pregnancy, only to return to normal at parturition in readiness for the control of maternal behaviour [346, 443]. This is supported by the evidence that not only application of a $GABA_A$ receptor antagonist, BM in this present study, but also administration of benzodiazepine antagonists is able to decrease aggression during lactation [237].

Examination of activated GAD65/67 cells after a maternal aggression test highlighted two brain regions where GABA neurotransmission may be acting to

control maternal aggression, namely the SON and MeA. In the SON, there was a decrease in the number of activated GAD 65/67 cells following maternal aggression. Within the SON there are a large number of $GABA_A$ receptors which inhibit OXT secretion [444, 454]. OXT, which is mainly secreted by the SON and PVN, is implicated as being essential for the control of maternal aggression [152, 188, 198, 227, 265, 268, 269, 282, 320, 431, 439, 474]. Thus the decreased activation of GAD 65/67 containing cells within the SON may indicate the disinhibition of GABA controlled OXT secretion within the SON to increase central OXT release to enable maternal aggression. Investigation of the effect of a direct application of $GABA_A$ receptor antagonist on central OXT secretion and maternal aggression would elucidate whether this hypothesis was correct or not.

Intracerebral infusions of BM into the amygdaloid complex of lactating rats reduces maternal aggression therefore the increase in activation of GAD65/67 cells in the MeA alone of lactating rats suggests this division of the amygdala may be important in the control of maternal aggression [390]. Future experiments should examine the effects of direct infusions of BM into this area on maternal aggression. It would be advisable to first perform a dose response curve to accurately identify the correct dose [475, 476]. GABA is the main inhibitory neurotransmitter in the brain and hence impacts on many different functions not just behavioural ones, therefore a dose response curve should be performed to ensure the dosage of GABA administered will cause a behavioural effect but no dangerous side effects such as motor impairments.

The effect of BM on maternal aggression was also examined in pup-sensitized virgin female rats to investigate whether the aggressive behaviour induced

by pup-sensitization is under the same GABAergic control as observed in lactating rats. However, only very low levels of maternal aggression were observed in pup-sensitized virgin female rats with no difference between the treatment groups. This suggests, as observed in chapter 2, that pup-sensitization alone it not enough to induce maternal aggression in the virgin rat and inhibiting GABA neurotransmission does not affect this.

Interestingly, there was a significant increase in time spent freezing in BM treated pup-sensitized virgin rats. As freezing is an expression of 'fear-like' behaviour, it is speculated that the GABA antagonist treatment could increase neophobia (fear of something new). This idea is further supported by the significant increase in Fos expression in the BnST following BM treatment, an area important in fear regulation [105]. This is in concordance with the effects of $GABA_A$ receptor agonists on neophobia behaviour in male rats; application of $GABA_A$ receptor agonists increased time spent in open arms of a the EPM whereas the GABA antagonist (BM) decreased it [477]. Research has observed the pup-sensitized virgins rats do display reduced neophobia typical of lactating rats, so application of a GABA antagonist may have dampened this reduced neophobia induced by the pups [174, 406]. To confirm this though, testing pup-sensitized virgin rats treated with a GABA antagonist in fear behaviour testing paradigms is required. One would predict treatment with a GABA antagonist would increase fear or neophobia compared to vehicle treatment.

However, no difference was observed between BM and vehicle treated lactating rats in the expression of freezing behaviour. Reduced freezing behaviour in lactating rats is thought to be partly due to the level of interaction with pups [42, 237,

390]. Therefore, the difference observed in anxiety behaviour display (freezing behaviour) between lactating and pup-sensitized virgin rats may be due to a lack of experience of specific pup behaviours which involve high levels of pup interaction such as suckling. Suckling is known to enhance GABA action, resulting in increased aggression [390].

This is interesting in relation to the data presented in the previous chapter. AP treatment reduced anxiety in lactating rats by potentiating $GABA_A$ receptor functioning proposed to be necessary for allowing aggressive behaviours to be expressed. This suggests that GABA may not only have a direct role over maternal aggression, especially certain offensive behaviours, but also indirectly through the actions of AP which enhance aggression by reducing anxiety via $GABA_A$ receptors.

In conclusion, GABA neurotransmission around the peri-partum period has many important functions. During pregnancy, its inhibition of OXT neurons ensures there is no early delivery allowing full development of the unborn pups. In addition, $GABA_A$ receptor potentiation through AP reduces anxiety in the pregnant mother preventing overstimulation of the HPA axis and therefore harmful steroids passing to the developing fetus [34, 36]. At term, disinhibition by GABA is essential for allowing the increase in OXT secretion to allow parturition and lactation to occur.

In terms of specific maternal behaviours, inhibition of GABA neurotransmission appears important for pup retrieval and nursing [416, 438]. However, for maternal aggression enhancement of GABA neurotransmission is required [158, 237, 390]. It may be that these two features of GABA neurotransmission are interlinked in controlling maternal behaviour. For example suckling has been shown to affect the pathways projecting from the peripeduncular

nuclei resulting in enhancement of GABA neurotransmission in the amygdala which may be necessary for the display of aggression [390]. This enhancement in the amygdala may in turn cause an increase in GABA neurotransmission to areas such as the MPOA which control maternal care, to switch off other maternal behaviours allowing expression of only maternal aggression. When aggression diminishes, the decrease in GABA neurotransmission in the amygdala turns off GABA neurotransmission to the MPOA allowing the expression of other maternal behaviours. The amygdala and MPOA are used as examples here because they are two brain areas with clear maternal behavioural functions linked with GABA neurotransmission; in the MPOA, which is important for maternal care, enhancement of GABA neurotransmission decreases pup related behaviours [416, 438]. In the amygdala inhibiting GABA neurotransmission results in a decrease in the display of aggression [390]. It also should be noted that both regions display an increase in activation in GAD65/67 cells following maternal behaviour (MPOA) and aggression (MeA) respectively. Such findings however do not tell us how these neurons are acting, they may be projecting an inhibitory input to another area or act locally [355]. Retrograde tracer injections would enhance our knowledge as to how and where these inhibitory neurons may be located.

GABA neurotransmission is therefore important and critical during the lactation period; it is required for both maternal behaviour and aggression. Maternal behaviour may be switched off whilst aggression is displayed with both being controlled by GABAergic pathways.

Chapter Seven: General Discussion

7.1 Maternal aggression: expression of behaviour

One of the main aims of this thesis was to build a complete picture of the changes in maternal aggression during the peri-partum period in the rat. Comprehensive examination of maternal aggression throughout the peri-partum period has provided data to construct Fig. 7.1a. This now allows for direct comparisons with mice in which maternal aggression through the peri-partum period is already clearly defined (Fig. 7.1b) [6, 54, 169]. Maternal aggression peaks during the first week of lactation in both rats and mice, however in rats just prior to parturition, a small peak in maternal aggression is observed whereas the intensity of aggression in mice remains low. On the day of parturition there is a dip in the intensity of maternal aggression before rising to its peak the day after. Maternal aggression in rats starts to recede earlier than in mice with diminishing maternal aggression levels observed from the start of lactation week two. This is not seen in mice until midway through lactation week two. The difference in the expression of maternal aggression between these rodent species highlights the possibility that neuromodulators may control maternal aggression differently between species.

This thesis has for the first time clearly defined the expression of each specific component of maternal aggression throughout the peri-partum period and also following pup removal (chapter 4 and 3 respectively). With these data, direct comparisons can be made between lactating rats under different experimental conditions. In this thesis, maternal aggression is observed to diminish in lactation day 21 rats to levels similar to those expressed by lactation day 7 rats (chapter 4), whose pups were removed for 24h (chapter 3; number of attacks $p=0.35$, $T_{(8,9)}=75.0$,

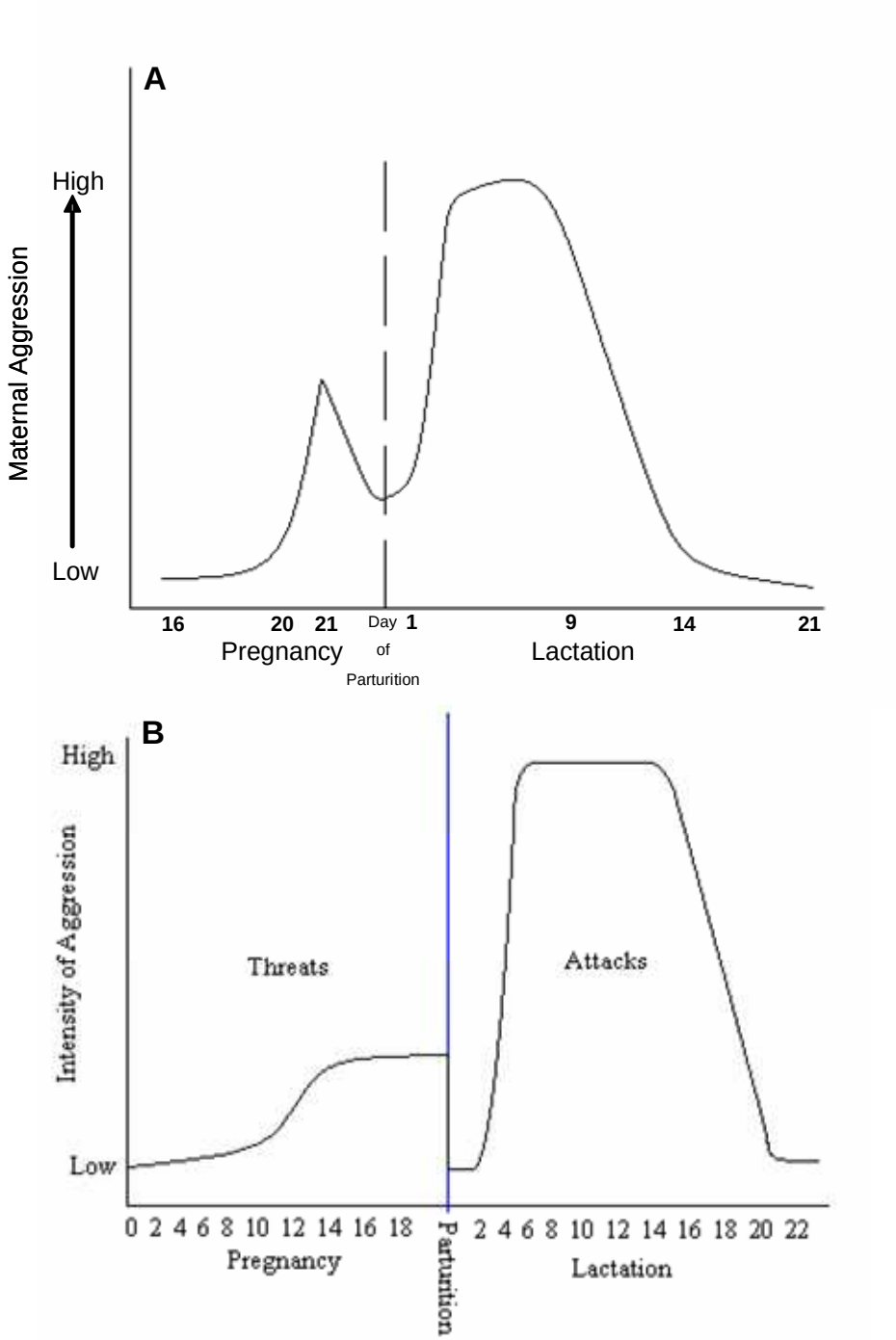


Figure 7.1: Maternal aggression levels throughout the peri-partum period in rats and mice. Schematic diagrams of the change in maternal aggression levels through pregnancy, parturition and lactation in rats (A) and mice (B). A is derived from data in chapter 3 of this thesis and B is modified from Gammie and Lonstein (2006) [54]. Maternal aggression in rats has a small peak during late pregnancy before dipping slightly on day of parturition and then intensifying for greatest maternal aggression expression during the first week of lactation. Maternal aggression then diminishes by lactation day 14 in rats. Mice, by contrast, show a gradual rise in maternal aggression during late pregnancy before dropping back down to very low levels on the day of parturition. Maternal aggression then reaches peak expression, like rats, in the first week of lactation before gradually diminishing by lactation day 20 slightly later than in rats.

latency to attack p=1.0, $T_{(8,9)}$=57.0). From this, one can propose that not only does the exhibition of maternal aggression depend on the stage of the peri- partum period the female rat is at, but also on significant input from the pups. This is important evidence to support the idea that the hormonal changes of pregnancy and parturition in the body and the brain 'prime' the future mother to respond to pup cues at parturition. These cues then maintain maternal aggression and other maternal behaviours through lactation [3, 44, 51, 56, 180, 184, 348, 400, 478]. Pup removal for 6h in this thesis resulted in significant reduction in maternal aggression; others have observed a significant decrease by 4h leading to the conclusion that between 4 and 6h after pup removal the mother switches off the ability to express maternal aggression.

The presence of pups has been proven to be necessary for lower neophobia and fear expression in both lactating and pup-sensitized female rats during anxiety and fear behavioural testing paradigms; reduced neophobia and fear is proposed to be essential for maternal aggression [42, 174, 210, 390, 406]. Many studies observe a decrease in anxiety when maternal aggression intensity is high in lactating mice and rats [29, 31, 32]. However, this reduced neophobia and fear phenotype is not always seen alongside high levels of maternal aggression. Therefore lower neophobia may only be essential at parturition in order to initiate the onset of maternal behaviour (a pre-requisite for maternal aggression) [15, 206-208, 400]. But once maternal behaviour and therefore maternal aggression begins, pup cues maintain maternal aggression so the lower neophobia phenotype is not a necessity [169, 179, 190]. Indeed, as discussed in the introduction of chapter 3, olfactory and sensory cues from pups are essential in maternal aggression maintenance with different regulatory

effects in mice and rats during lactation [169, 179, 184, 188, 190, 225, 400]. This does not rule out the possibility that there may be species or strain differences. Therefore further research is still needed to investigate the true link between fear, neophobia and maternal aggression in the lactating rodent.

Removal of pups for 24h results in maternal aggression levels similar to those observed on lactation day 21, but little is known about what changing cues from pups causes the natural decline of maternal aggression. Pup cues must be able to regulate the intensity of maternal aggression as lactation day 8 rats who fostered 18 day old pups expressed lower maternal aggression compared to lactation day 8 rat with 8 day old pups [191]. This indicates that cues from pups must contain information about their age, or more likely 'independence' and it is this which is crucial in controlling the intensity of maternal aggression. Olfactory cues are also expected to play a role. One way to examine this would be to observe maternal aggression in rats late on in lactation whose pups were immobilised. If unchanging olfactory cues were the most important factor then there would be no change in the intensity of maternal aggression. If physical and visual cues are important then one would expect mothers whose pups were mobile to express significantly less maternal aggression than mothers whose pups were immobile.

Input from pups is essential in maintaining maternal aggression and preventing the switch off of maternal aggression until the required time (i.e. late lactation), but where the pup cues act in the brain is as yet undefined. Pup removal for 2h resulted in significantly lower Fos expression within the BnST, MeA, SON and PVN indicating these regions may be highly sensitive to pup cues. Furthermore, significant positive correlations between Fos expression in these brain regions and

the time spent expressing specific maternal aggression behaviours are further evidence that pup cues may work through these brain regions to regulate maternal aggression (section *7.3* will discuss further how these brain regions are proposed to be involved in the maternal aggression neuro-circuitry).

This thesis describes observations, along with previous research, that although maternal behaviour is easily induced in virgin female rats by constant exposure to pups, maternal aggression is not [10, 174]. Pup-sensitized virgins expressed most if not all of the specific maternal aggression components in both the GABA antagonist experiment (chapter 6) and pup removal experiment (chapter 3), however the observed levels are only comparable to dams with pups removed for 24h or lactation day 21 dams. Yet, previous studies have observed that pup-sensitized virgins express a fear and neophobia profile similar to lactating rats when their pups are present [42, 174, 390, 406]. Furthermore, Fos expression in the PAG and PVN of pup-sensitized virgin rats with pups present during maternal aggression testing was not significantly different from lactating rats with their pups present (chapter 3). Thus pup-sensitized virgins display lower fear and neophobia levels (and more activation in brain regions which control fear and neophobia) proposed to be essential for enabling maternal aggression, but some other component is missing, hypothesised to be the hormonal influences of pregnancy, preventing the full expression of maternal aggression.

Previous research has shown the importance of ovarian hormones; if a pup-sensitized rat is treated with estrogen and progesterone to mimic pregnancy, attack behaviour increases [53]. Indeed, pup-sensitization for 18 days will induce higher levels of maternal aggression; this long period of pup-sensitization induces a

pseudopregnant state at the time of maternal aggression testing [172]. Pseudopregnancy causes hormonal changes similar to those observed during pregnancy hence this could explain why the higher level of maternal aggression is observed [172]. However, because the full suite of maternal aggression behaviour was not observed, and a lengthy pup-sensitization period is required before maternal aggression is expressed, other neuromodulators must also have an important role. Evidence for which particular neuromodulators may be involved in regulating maternal aggression is discussed in the next section but the pup-sensitized virgin rat model provides an excellent means to examine the relationship between neuromodulators and maternal aggression. The pup-sensitized virgin rat does not experience the hormonal changes of pregnancy (unless exposed to pups for a long time); so they will be expected to have baseline circulating levels of hormones and neuromodulators. Thus the effect of administering a specific neuromodulator either brain wide or to specific brain regions on maternal aggression expression can be examined.

7.2 Maternal aggression: neuromodulator control

Oxytocin

From the summary figures 7.2 and 7.3, it can be observed that there are significant changes in the OXT, AVP, AP or GABA systems in the brain following maternal aggression testing.

Many changes are observed in the central OXT system that are correlated with maternal aggression in the lactating rat. Fig 7.4 summarises the changes in the OXT system in different brain regions in an aggressive lactating rat from data

collected during this thesis. OXT is therefore important in the control of maternal aggression but the mechanisms by which OXT works to regulate maternal aggression still remain uncertain.

OXT has a great influence over the HPA axis activity and was proposed to be important for the blunted HPA axis response to a stressor during lactation [126-129, 227],[35]. However, ICV OXT antagonist administration has no effect on the HPA axis response to stressors in the lactating rat, but OXT antagonist treatment does affect their anxiety behaviour [35]. Hence OXT may enable maternal aggression to be displayed by reducing fear but the link between fear, anxiety and maternal aggression needs to be investigated further. However, there is evidence from the established changes within the OXT system at parturition that could account for the reduction in fear and anxiety necessary for the onset of maternal behaviour. In this thesis, OXT binding is increased in the BnST and MPOA on day 21 of pregnancy and previous research has shown that OXT antagonist treatment prevents the onset of maternal behaviour [131, 316, 317]. The BnST and MPOA are essential for driving maternal behaviour control because direct lesions in these brain regions result in impairment of all maternal behaviour [27, 67, 79]. OXT may therefore work in or through the BnST and MPOA to reduce fear and anxiety to enable the mother to approach her pups as soon as they are born, and express maternal behaviour.

Increased OXT secretion in the PVN on day 19 of pregnancy was observed only during a maternal aggression test. The pregnant rat therefore may increase OXT secretion to reduce fear and anxiety in response to a stressor but examination of OXT secretion in the PVN of a virgin rat during a resident-intruder paradigm is required to confirm this. This release of OXT could also be important to prevent HPA axis

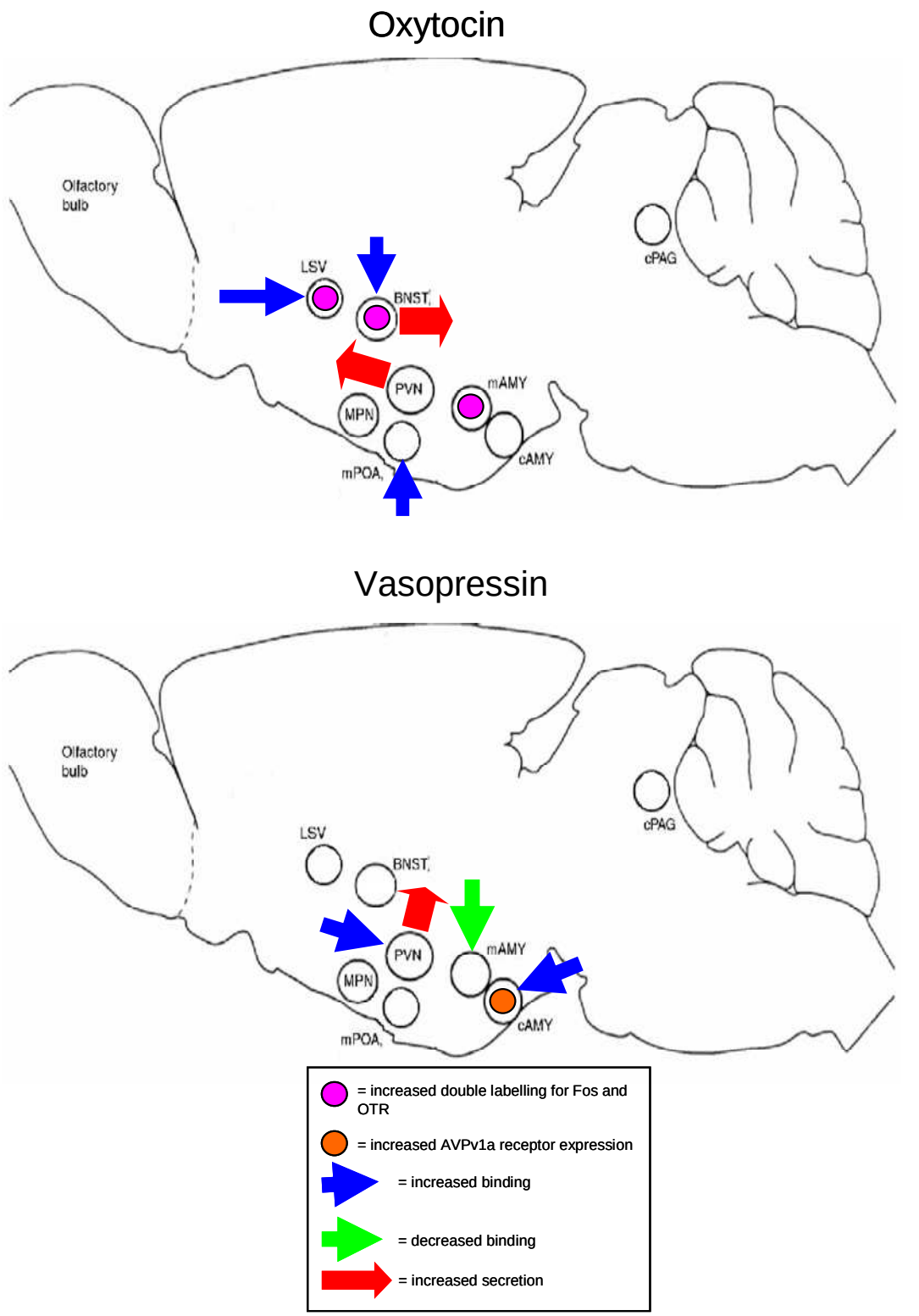


Figure 7.2: Changes in the oxytocin and vasopressin systems in specific brain regions of the female rat after maternal aggression testing. Figure A depicts the significant alterations in Fos and OTR expression, oxytocin binding and secretion in specific brain regions of the female rat during the peri-partum period after maternal aggression testing. Figure B shows the different changes in the vasopressin V1a receptor mRNA expression, vasopressin secretion and binding in specific brain regions of the lactating rat after maternal aggression testing.

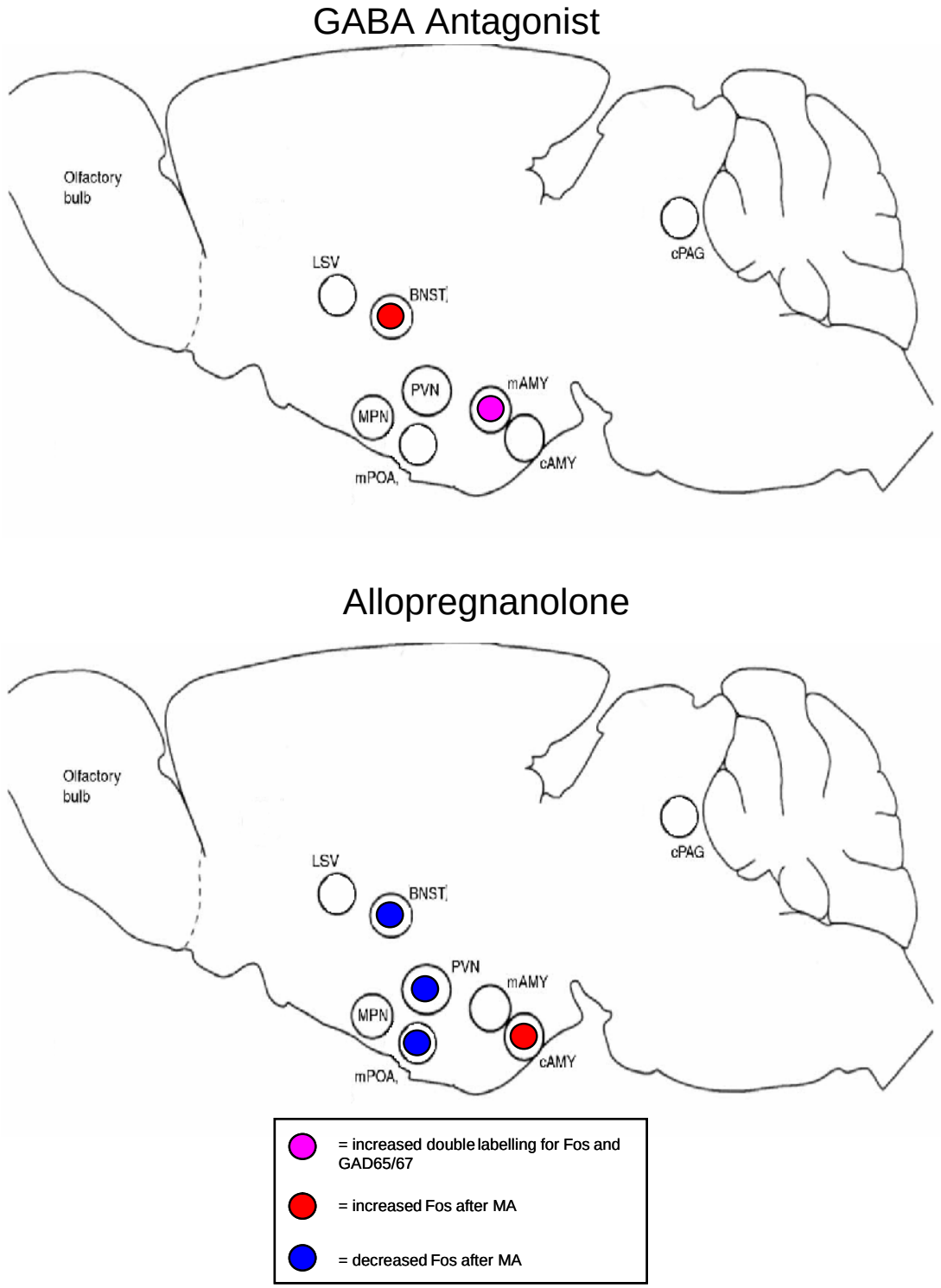


Figure 7.3: Changes in the GABA and allopregnanolone systems in specific brain regions of the female rat after maternal aggression testing. Figure A depicts the specific brain regions which exhibited a significant higher number of Fos and GAD 65/67 cells in the female lactating rat after maternal aggression testing. Both figures depict the specific brain regions which showed significant changes in Fos expression (higher or lower) after application of a GABA antagonist or AP (figures A and B respectively) in the lactating or pup-sensitized virgin female rat after maternal aggression testing.

activity so that the developing fetus is not exposed to the deleterious effects of glucocorticoids. However, no changes in OXT binding in the PVN or PAG were observed during the peri-partum period, two regions known to be important in expression of fear and anxiety and regulation of the HPA axis. Therefore, further research should focus on where and how OXT may act to induce a reduced fear and anxiety phenotype at the correct time during the peri-partum period.

Furthermore, this action of OXT in reducing fear and anxiety pre-partum to enable maternal aggression post-partum does not explain why there is an increase in OXT secretion in the BnST and PVN during maternal aggression testing on lactation day 4. Or why increased Fos expression was observed in OXT sensitive neurones in the BnST, LS and MeA of the lactating rat during maternal aggression testing. As OXT is not modulating the HPA axis response to the intruders which may be viewed as a stressor, this increased OXT secretion must have functions elsewhere in the brain [35]. The PVN and BnST are known to project to the PAG and LS which has OXT sensitive neurones and hence may OXT influence the actions of the motor control aspect of maternal aggression [90, 308, 479-483].

There is an increase in the activation of OTR containing cells in aggressive lactating rats in the LS, but no difference was found in the PAG. However further research is required before the idea that OXT may modulate the motor control of maternal aggression can be totally ruled out, as non-aggressive lactating rats was still performing other maternal behaviours which may still require the action of the OXT sensitive cells in the PAG. Therefore, investigation of OXT secretion in the PAG by microdialysis during a maternal aggression test in the lactating rat is required. Also, by further experiments that examine the effects of direct OXT or OXT antagonist

treatment into the PAG on the display of maternal aggression would establish if this theory that OXT can modulate the motor output of maternal aggression is correct.

In the LS, OXT secretion does not change during maternal aggression testing despite an increase in OXT binding on lactation day 4-7 and increase in Fos activation in OXT sensitive cells. This suggests that OXT sensitive neurones that are important for the maternal aggression circuitry may project through the LS. Tracer studies would enable the identification of where these OXT projections come from. Recently, direct administration of a GABA antagonist to the LS decreased maternal aggression in mice suggesting that GABA functioning in the LS may directly influence upon maternal aggression [387]. However, no changes in maternal aggression or Fos activation in the LS were observed after AP or finasteride treatment suggesting that GABA neurotransmission has no effect on maternal aggression in the lactating rat. Although this does not rule out that the possibility this GABA may function in the LS to increase maternal aggression and this may be independent of AP influences. Nonetheless, there were no differences in Fos activation in GAD65/67 containing cells in the LS between aggressive and non-aggressive lactating rat. This could be an example of a neuromodulator controlling maternal aggression differently in individual species as in mice direct application of BM to the LS significantly reduces maternal aggression; to confirm this investigation of the effect of a GABA antagonist directly applied to the LS of a lactating rat on maternal aggression is required.

Allopregnanolone and GABA

One neuromodulator that may control the blunted response of the HPA axis to stress during lactation is AP. Although there were only minor effects observed on

maternal aggression after AP or finasteride application, Fos expression was significantly lower in the PVN and PAG of lactating rats following a maternal aggression test. This reduction in Fos expression, especially within the PVN, could reflect the action of AP preventing the activation of the HPA axis in response to the intruder. During pregnancy, AP is known to prevent a HPA axis responses to an immune stressor [199]. Research should therefore focus on investigating if AP has a direct effect on the HPA axis response to a stressor during lactation. This could indirectly enable maternal aggression, therefore the actions of AP in influencing maternal behaviour directly or indirectly still warrants further investigation.

One way in which AP may directly modulate maternal aggression is through its actions on $GABA_A$ receptors which are already established in regulating neophobia and fear [356-359, 361]. Subcutaneous AP administration to lactating rats before maternal aggression testing resulted in a decrease in Fos expression in the BnST and an increase in attack number. The BnST is involved in the long term control of fear so the decrease in Fos expression may reflect inhibition of the BnST by GABA to attenuate fear allowing increased maternal aggression [105]. In pup-sensitized virgin rats, however, bicuculline methiodide (BM) treatment caused an increase in freezing (indicating fear) and an increase in Fos expression in the BnST compared to vehicle treated pup-sensitized virgins during a maternal aggression test. This suggests that GABA neurotransmission inhibition increases fear in conjunction with an increase in BnST activity [105]. Examining the effects of BM treatment to the pup-sensitized rat in anxiety behaviour paradigms would test if this theory is correct. To show it is the actions of AP controlling GABA actions on fear, testing the

effect of direct AP injections into the BnST prior to testing fear responses in the lactating rat is required.

Administration of AP also increased Fos expression within the CeA, an area well established as part of the fear circuitry [84, 95, 375, 423, 440, 484, 485]. Thus, AP may directly influence maternal aggression by regulating fear in the BnST and CeA through its effects on $GABA_A$ receptors. However there is substantial evidence linking OXT actions in the CeA to maternal aggression whereby only a small effect of AP administration was observed. One could propose though that AP may provide a backup to OXT through its ability to manipulate GABA actions within the BnST and the CeA. Thus future research should investigate the effects of applications of GABA, AP and OXT agonists and antagonists directly into the CeA to establish if this theory is correct.

Vasopressin

AVP plays an important role in regulating maternal aggression. As proposed in the discussion of chapter 4, the actions of AVP on maternal behaviour are the opposite to OXT. AVP binding decreased on pregnancy day 21 in the MeA, a region important for processing olfactory information [30, 70]. Olfactory cues from pups processed by the MeA are proposed to inhibit aggression in virgin rats [30, 70, 89, 98-100]. Pregnancy day 21 is the time when maternal responsiveness increases, hence the decrease in AVP binding may reflect the switch in MeA processing of olfactory inputs from pups as fearful to salient to enable maternal behaviour immediately after parturition. Interestingly, AVP binding in the CeA was increased at parturition as well as increased AVP V1a receptor mRNA expression. The CeA is involved in short term fear regulation so this may reflect a transient increase in fear

of the intruder preventing maternal aggression, but instead allowing the lactating rat to focus on her pups to create a strong mother pup bond [105]. This is proposed because on the day of parturition a drop in the level of maternal aggression is observed in the lactating rat.

All four (OXT, AVP, AP and GABA) neuromodulators studied in this thesis have an important role in regulating maternal aggression but the timing of their actions is critical and is evoked in response to specific stimuli. It is of course important to remember that these four neuromodulators are not the only cues involved in the regulation of maternal aggression as it is already established that prolactin, serotonin and dopamine are all implicated [188].

7.3 Maternal behaviour and maternal aggression: brain circuitry

There are many brain regions indicated as being involved in the highly complex maternal aggression circuitry (see general introduction) and the results of this thesis and previous research lead to the proposed maternal aggression circuitry summarised in Fig. 7.4.

The hormonal experience ‘primes’ the rat to express maternal behaviour. This is required before maternal aggression can be expressed [15, 206-208, 400] . In the PVN and SON, the hormonal changes of pregnancy result in a hyporesponsive HPA axis to stressors and a reduced fear and anxiety phenotype [37, 38, 135-137]. This reduced fear and anxiety is important as it enables the dam to approach pups immediately after parturition and hence initiate maternal behaviour. The PVN may centrally control the expression of fear and anxiety by regulating the activity of CeA and PAG. Projections from the PVN to the PAG are linked with the control of fear

and anxiety during lactation [308, 479]. These projections are related to the OXT system which links significantly with maternal aggression as discussed above [308, 479]. Links between the CeA and fear expression are well established in connection to the OXT system [84, 95, 423, 440, 484, 485].

The action of the MPOA is crucial to the onset of maternal behaviour [27, 67, 79-83]. However evidence from this thesis indicates the MPOA does not play a direct role in the regulation of maternal aggression. No difference was observed in Fos expression following pup removal when maternal aggression diminishes over time. Instead significantly higher levels of Fos expression were observed in pup-sensitized virgin female rats that spent longer time nesting than lactating dams during maternal aggression testing. Furthermore, Fos expression within the MPOA was not significantly correlated with the expression of any specific component of maternal aggression. Yet, previous studies have shown significantly higher Fos and other IEGs expression within the MPOA following maternal aggression testing [12, 194]. This may indicate that the MPOA is required to switch off other maternal behaviours to allow the full suite of maternal aggression behaviours to be displayed, and therefore this means that the MPOA may have an indirect role in maternal aggression regulation. Further research is required to test this theory, this would best be done through direct MPOA application of specific neuromodulators as direct MPOA lesions significantly disrupt maternal behaviour. Hence it would be hard to deduce whether the effects of the lesion on maternal aggression are due to poor maternal behaviour or direct impairment of maternal aggression.

The MPOA may be central to the control of other maternal behaviours, but the BnST may be the brain region at the heart of the maternal aggression neuro-

circuitry. Fos studies reveal that the BnST is highly activated during a maternal aggression test and Fos expression within the BnST was significantly lower 2h after pup removal. In addition, correlations between Fos expression and expression of specific maternal aggression components found the BnST to be positively related to most aspects of maternal aggression. The hypothesis that the BnST is central to the maternal aggression is still in its infancy and as indicated on Fig. 7.4 there are many connections between the BnST and other brain regions in relation to maternal aggression yet to be established.

The PAG may control fear and neophobia, but positive correlations of Fos expression with the expression of specific maternal aggression behaviours clearly indicate it too has an important direct role in maternal aggression [141-145]. The PAG regulates motor function in the brain and hence one could propose that the BnST receives olfactory information from pups via the MeA and passes that information onto the PAG which activates the motor aspect of maternal aggression [30, 70]. However, pup removal reduces Fos expression within the PAG after as little as 6h indicating the PAG is also sensitive to pups cues. As PAG lesions significantly disrupt nursing, licking and retrieval behaviour all of which involve sensory input from pups, it could be proposed that sensory information from the pups (also important in maintaining maternal aggression) passes through the PAG to the BnST which then process all the information before it drives maternal aggression [90-92, 140].

These may seem to be conflicting roles for the BnST and PAG in that they both receive pup cues and are also directly regulating maternal aggression. This thesis, however, has only examined the BnST and PAG as whole regions. Like the

amygdala, both these regions can be divided into sub-regions and it may be that within specific sub-regions of the BnST and PAG that these proposed functions of the BnST and PAG are controlled.

The MeA has links with both the inhibition and facilitation of maternal aggression [10, 12, 70, 98, 188]. Many studies observe a higher activation in the region following a maternal aggression test in lactating rats and mice [12, 188, 194, 196]. This was also observed in this thesis in the AP and maternal aggression and the maternal aggression and EPM experiments (chapters 5 and 3 respectively). Furthermore in this thesis, the level of Fos expression was significantly correlated with many specific aggressive components of maternal aggression. Therefore, there must be some switch that occurs in the MeA which changes it action from inhibiting maternal behaviour to enabling maternal aggression. It would appear that this influence is likely to be hormonal as Fos expression in lactating rats following a maternal aggression test was only significantly lower 24h after pup removal. This is proposed to be under the control of AVP as discussed in section *7.2*. However, it is important to note that pup cues influence MeA activity as pup exposure alone will induce maternal behaviour in naïve virgin female rats hence overcoming the MeA inhibition [10, 70, 98].

The MeA is involved in the flow of olfactory information to the MPOA and BnST from the OBs and hence is likely to be the crucial site for the processing of olfactory information from the pups to enable maternal aggression and maternal behaviour in lactating rats but prevent it in virgin female rats [70, 98, 402, 403]. Lesions of the OBs are able to significantly reduce the latency for virgin female rats to become ‘fully maternal’ during the pup-sensitization process although the MeA is

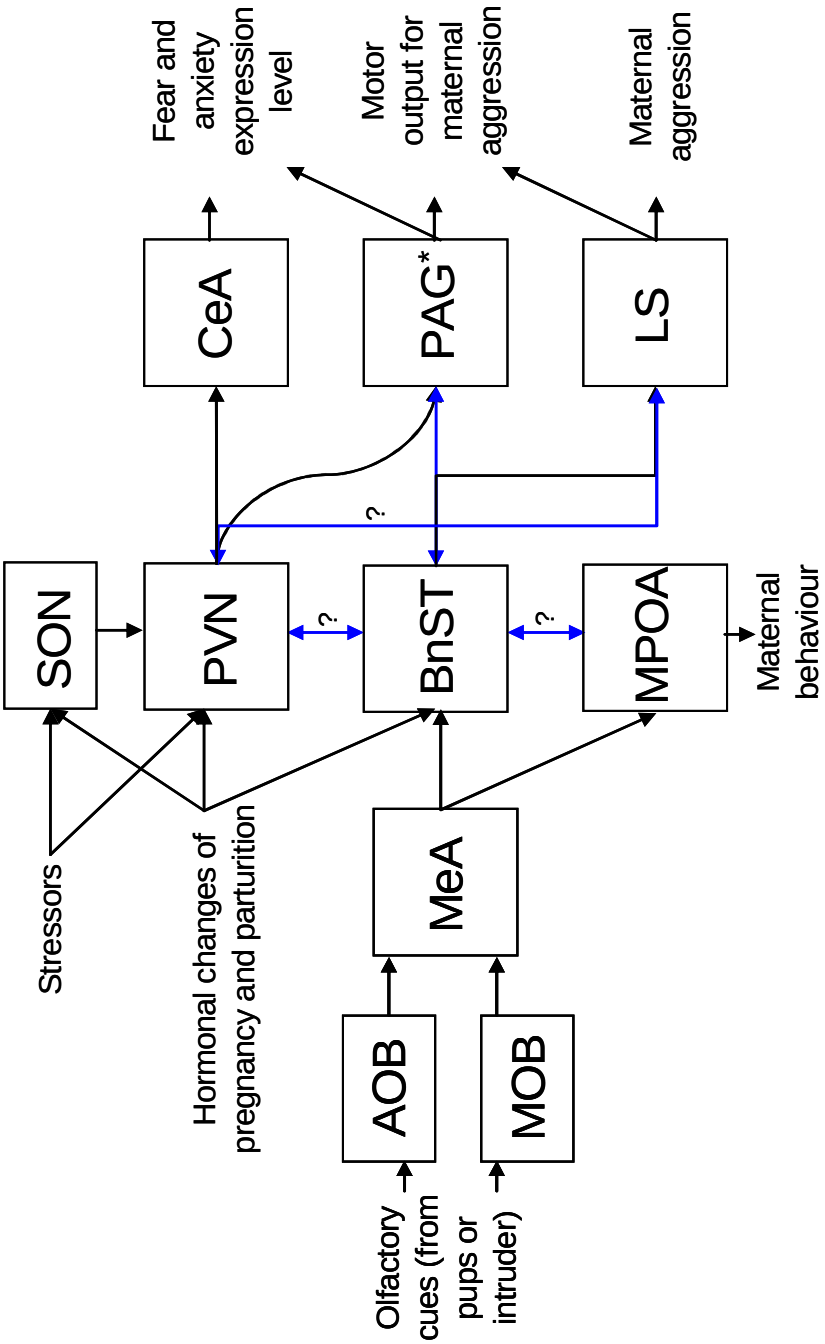


Figure 7.4: Maternal aggression circuitry. The flow chart depicts how the specific brain regions of the female rat brain are interacting to control maternal aggression during the lactation period. Firstly hormonal changes of pregnancy and parturition make the PVN and SON hyporesponsive to stressors. These regions project to the CeA and PAG to create a low anxiety and fear profile in the lactating rat. Pregnancy hormonal changes also prime the BnST to respond to information coming from the pups to enable maternal aggression by activating the LS and PAG areas. The cues from pups can be olfactory in nature, so will pass through and be processed by the olfactory bulb and MeA before reaching the BnST. There are also sensory cues (*) and these may be processed in the brain by the PVN before being passed along to the BnST. Connections in black are already established but connections depicted in blue are as yet to be clearly established in relation to maternal aggression.

indicated as involved this has not been proven [4, 30, 486].

The LS is one area whose role in the maternal aggression circuitry remains unclear. Fos expression is increased during maternal aggression testing in the lactating rat and this expression positively correlates with the expression of specific maternal aggression behavioural components. One current theory regarding the function of the LS is that it acts as ‘an effector’ in the maternal aggression circuitry, i.e. it enables maternal aggression, rather than ‘a mediator’ which controls maternal aggression [151]. The LS receives projections from the PVN and projections between BnST and MeA pass through the LS. Thus the LS could receive information from these regions informing it what component of maternal aggression to allow.

7.4 Concluding remarks

The circuitry for maternal aggression and maternal behaviour is highly complex and integrates many different brain areas which are involved in controlling the motor, motivational, fear and anxiety outputs (Figs. 7.2-7.4). This thesis has started to piece together how these brain regions influence each other; currently one would propose that the greatest influence on the neural circuitry comes from OXT and AVP which work oppositely to control maternal aggression by influencing by level of fear and anxiety in (Fig. 7.2). OXT is also indicated in control of the motor functioning of maternal aggression. Evidence in this thesis also implicates the involvement of AP and GABA especially in relation to fear regulation (Fig. 7.3). However, it remains unclear if these work in conjunction with OXT to enable maternal aggression or if they are a back up mechanism for OXT secretion malfunctioning. One hopes future research will be able to elucidate how these all integrate and impact upon each other to control maternal aggression and maternal behaviour.

By understanding the maternal aggression neural circuitry and the influences of neuromodulators on its regulation, would provide a means to manipulate maternal aggression by administration of specific neuromodulators. This knowledge could then be applied to the human mother and has the potential to create therapies for mothers who experience peri-partum disorders (for example postnatal depression) which disrupt their maternal care. Prevention and treatment of these disorders is not only beneficial to the mother to enable her to care for her offspring, but is also crucial for her offspring's development to prevent adulthood disorders stemming from their early life experience. Such experiences can have a dramatic effect on their own ability to demonstrate paternal or maternal care ability.

Appendix One: Brief description of expression changes of maternal behaviour components during lactation period.

Pup retrieval is immediately expressed in dams at a high rate at parturition, it declines between lactation day 12 and 16 when pups start opening their eyes and become mobile [1, 2, 7]. For proper expression of pup retrieval, rats require attachment to a stable nest area, as manipulation of ecological conditions (e.g. temperature, light, air current or crowded social conditions) can result in movement of the nest location. This causes scattering of the pups and a decline in maternal care that may result in pup death [1]. Nursing is first expressed once all pups are born and cleaned and the nest is completed [1]. It generally starts with the dam licking the pups to induce them to attach to the presented nipple which will enable milk ejection. Nursing continues for two to three weeks but by the third week pups are gradually weaned [487]. During weaning, when pups try to initiate feeding the dam will prevent any access to mammary glands forcing pups to search for alternative food sources [1]. Nest building is the one maternal behaviour which normally occurs before parturition. As the dam prepares for birth an area will be cleared with small walls of bedding surrounding [1, 487]. Once parturition has occurred the nest is then fully formed and maintained until the second week of lactation when the pups become mobile and start to venture from the nest [1]. By the third week, there is no definable nest area as the large pups will generally huddle in their own groups around the cage. Pup licking and grooming appears immediately after birth of the first pup.

Appendix Two: Brain maps

Below are rat brain maps, from The Rat Brain in Stereotaxic Coordinates by G. Paxinos and C. Watson, illustrating brain regions analysed during the studies.

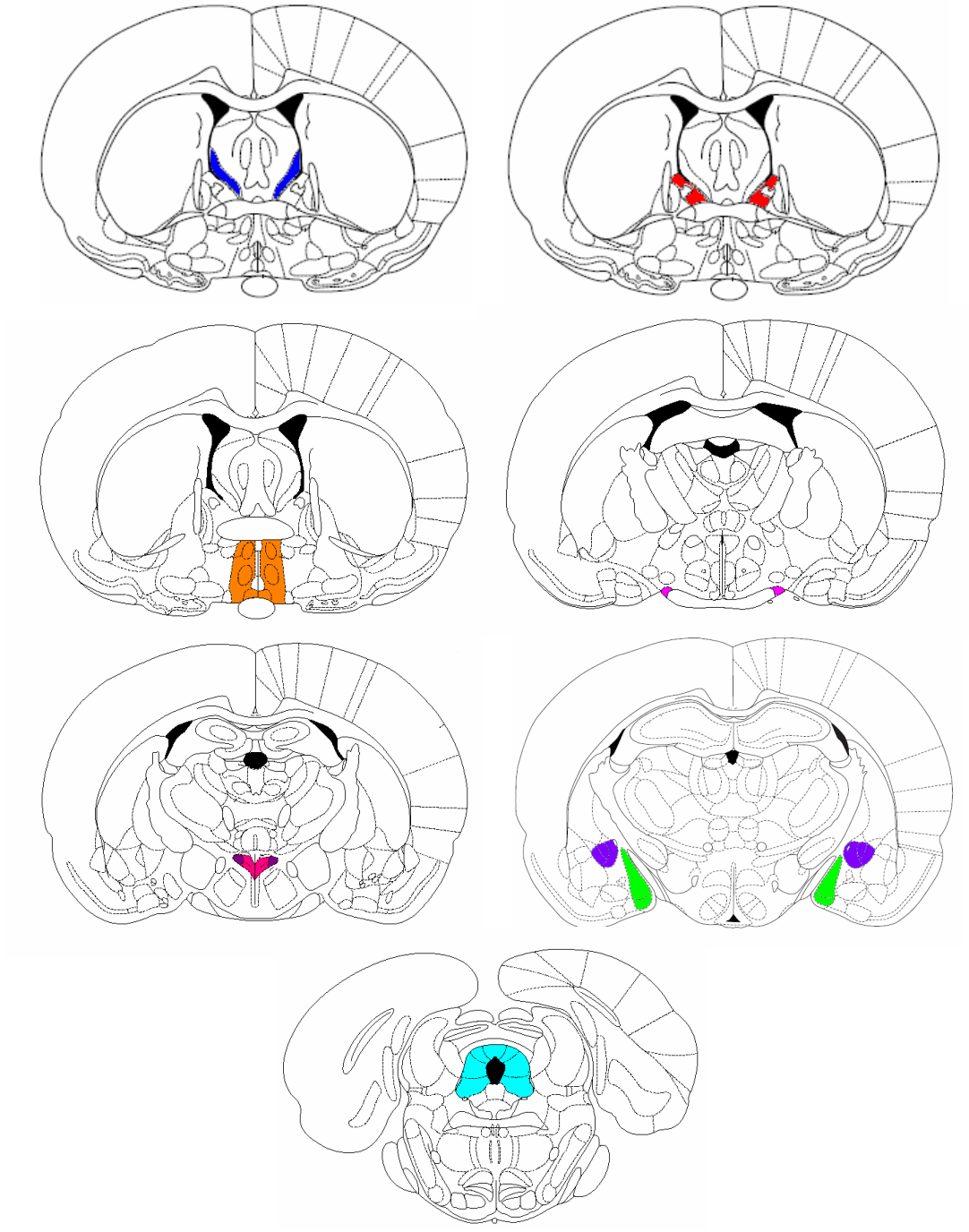
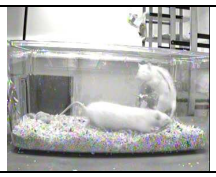


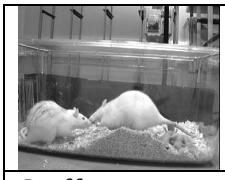
Figure 1: Rat brain maps highlighting specific brain regions. Highlighted in the rat brain diagrams are the lateral septum (blue), bed nucleus of stria terminalis (red), medial preoptic area (orange), supraoptic nucleus (pink), paraventricular nucleus (parvocellular – fuchsia, magnocellular – dark purple), amygdala (medial – green, central – purple) and periaqueductal grey area (turquoise).

Appendix Three: Behavioural photographs

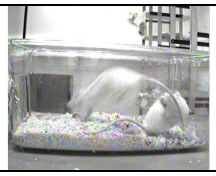
The following pictures are examples of the different types of behaviour scored using Noldus Observer Video Pro Version 5. The intruder is marked by black stripes on the back.

Aggressive Behaviour:

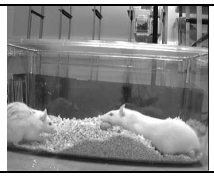
			
Attack	Biting	Clawing/Punching	Pinning Down

		
Sniffing	Lunging	Rearing

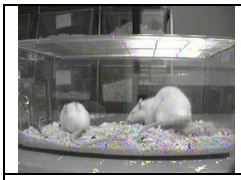
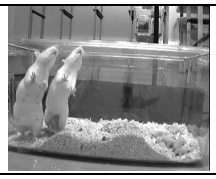
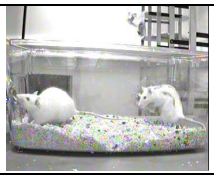
Response to Aggressive Behaviour:

		
Freezing	Escape	Rearing Away

Pup-related Behaviours

				
Pup Moving	Nursing	Nesting	General Pup Behaviour	Grooming

Other Behaviours

				
General	Exploring	Drinking	Eating	Grooming Self

Appendix Four: Behavioural analysis programs

The graphical user interface used by the observer for analysing the behavioural videos. Figure 1 depicts the interface for Observer 5.0 and figure 2 for Observer XT 7.0 used for analysing maternal behaviour or aggression videos. Figure 3 displays the screen used to setup the arena profile for EPM analysis (figure 4).

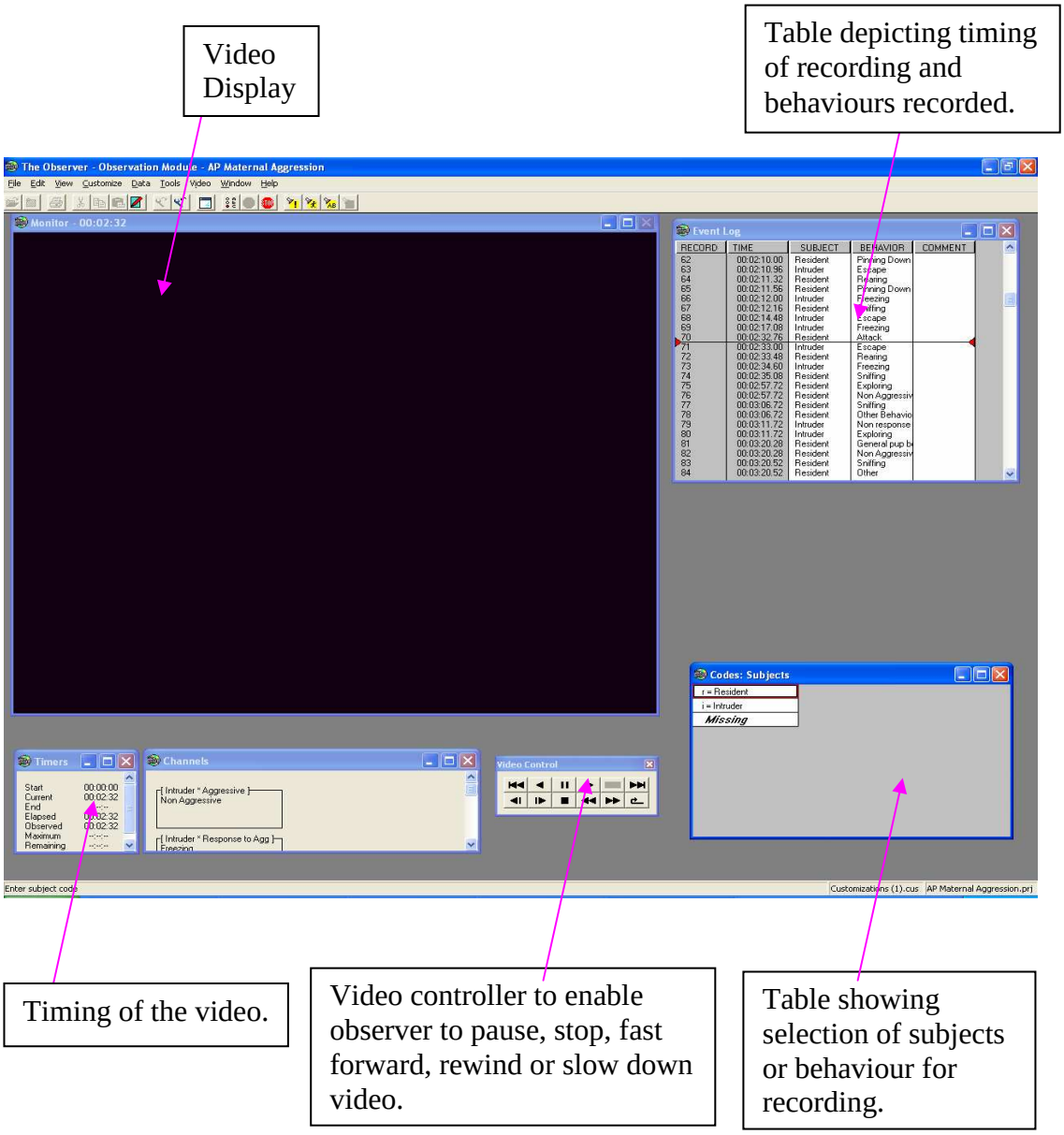


Figure 1: Observer Version 5.0 Behavioural Analysis Screen

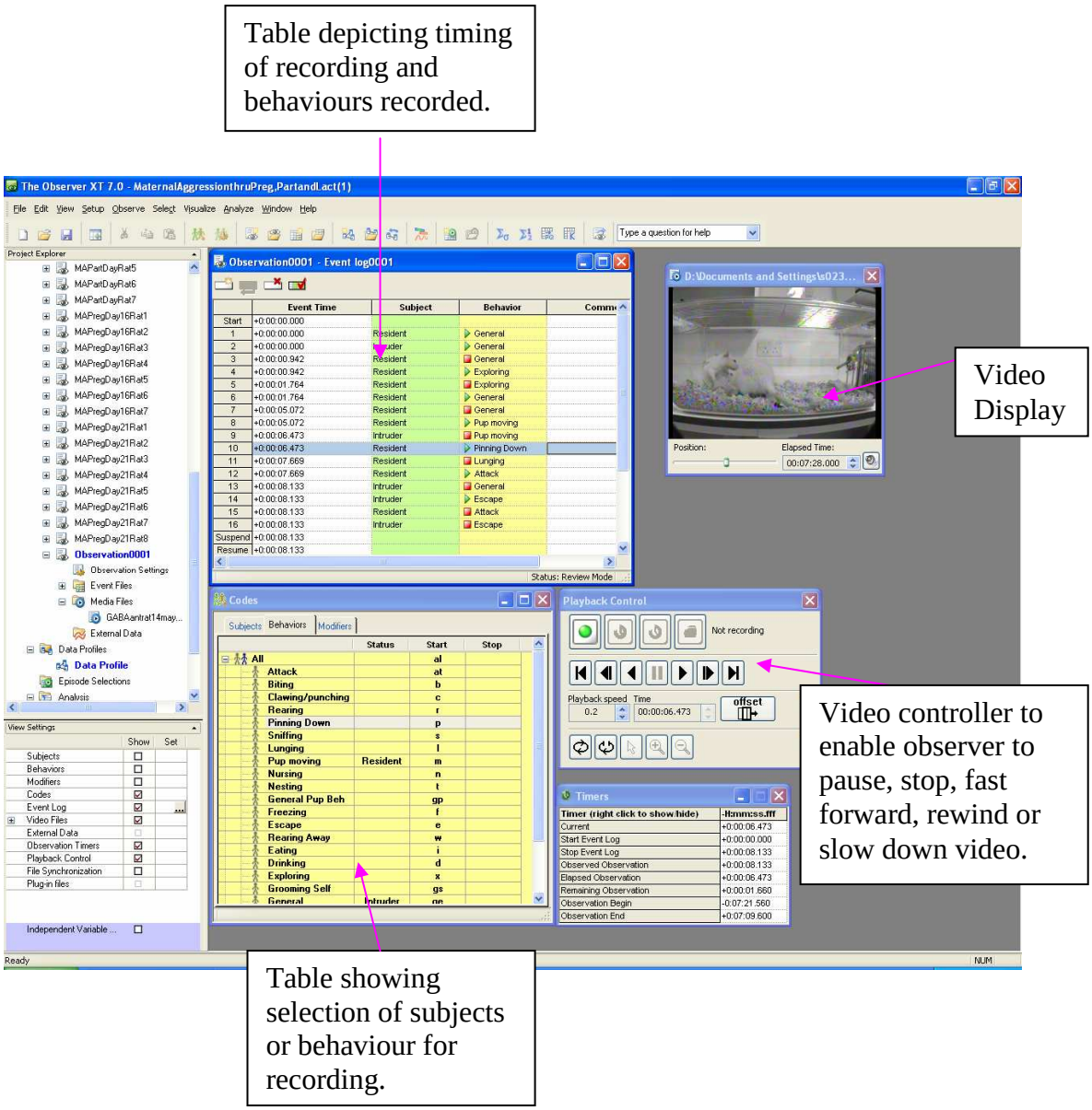


Figure 2: Observer Version 7 Behavioural Analysis Screen

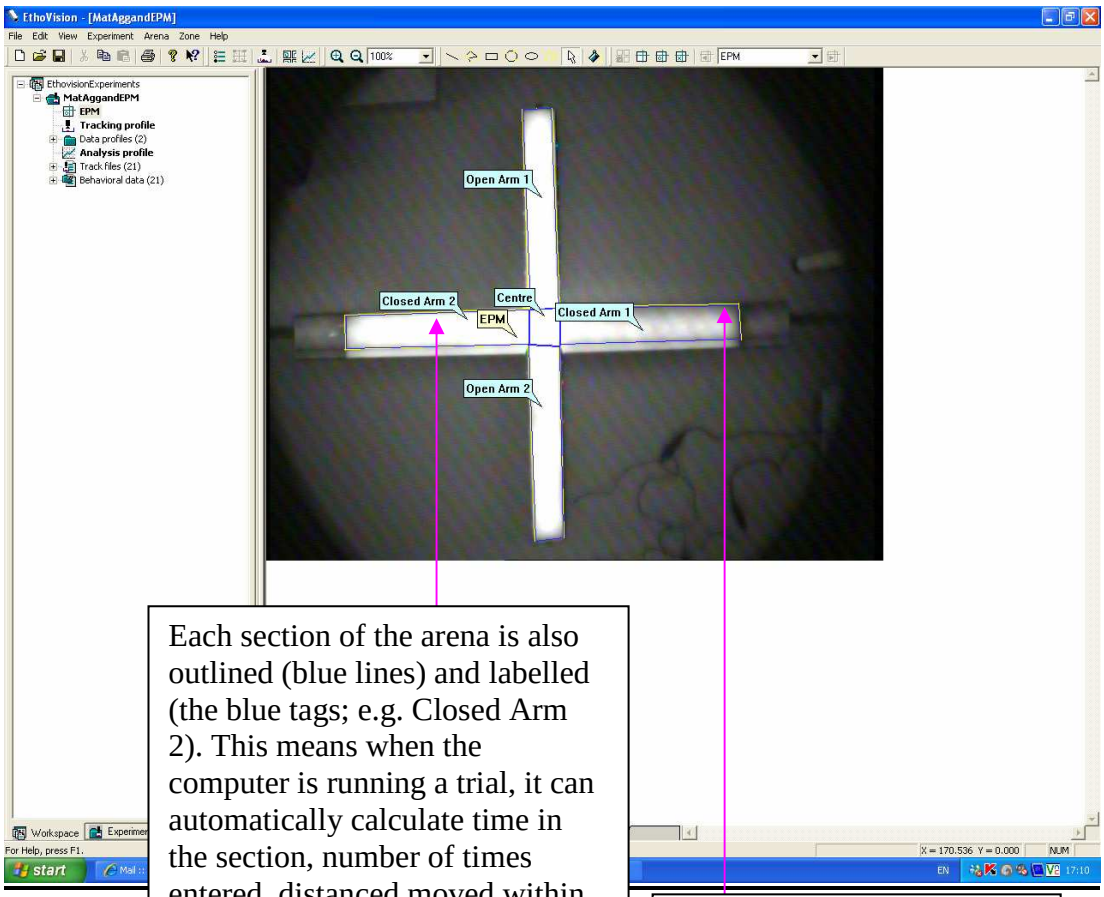


Figure 3: Arena Profile Setup

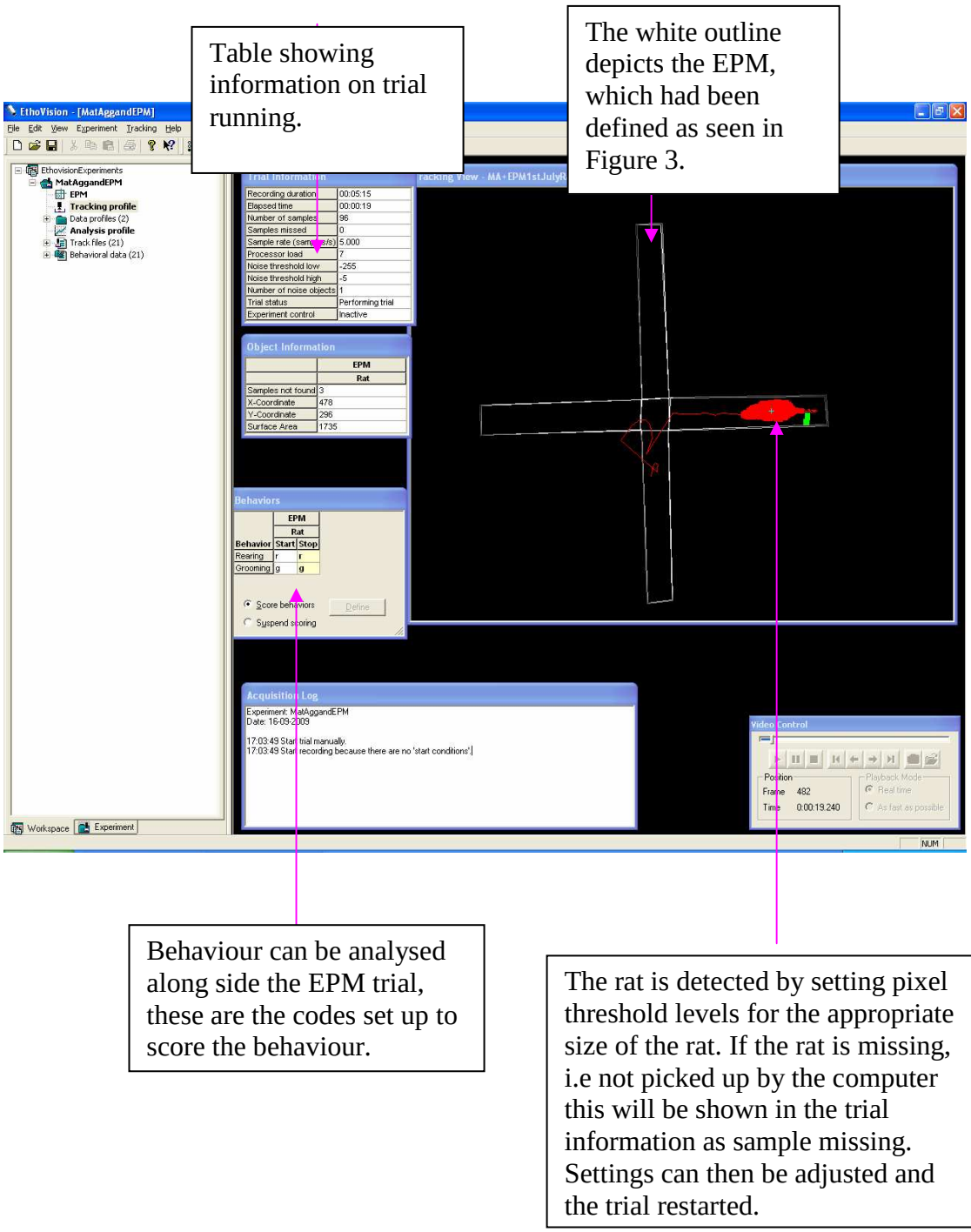


Figure 4: Trial Setup for analysis of Behaviour on the Elevated Plus Maze

Appendix Five: Solution recipes

Recipes for solutions in materials and methods chapter:
(All reagents were made using glass distilled water)

Acetic Alcohol Fixative

10ml	40% Formaldehyde
5ml	Glacial (100%) Acetic Acid
85ml	Absolute Ethanol

Heparinised Saline

1000ml	Glass Distilled water
9g	NaCl
129mg	Heparin (Sigma)

4% Paraformaldehyde in 0.1M Phosphate Buffer

40g	Paraformaldehyde
11.5g	Disodium hydrogen orthophosphate
2.72g	Sodium dihydrogen orthophosphate
1000ml	Distilled water

15% sucrose in 4% Paraformaldehyde in 0.1M Phosphate Buffer

15g sucrose per 100ml of solution above.

30% sucrose in 4% Paraformaldehyde in 0.1M Phosphate Buffer

30g sucrose per 100ml of solution above.

1M Phosphate Buffer (PB; pH7.4):

115g	Disodium hydrogen orthophosphate
27.2g	Sodium dihydrogen orthophosphate
1000ml	Distilled water

0.1M PB (pH7.4) with 0.3% Triton X-100 (PB-T):

3ml	Triton X-100
1000ml	0.1M PB

0.3% Hydrogen Peroxide:

99ml	0.1M PB
1ml	30% Hydrogen Peroxide

1% Normal sheep serum:

99ml	PB-T
1ml	Normal Sheep Serum

0.2M Sodium Acetate (pH 6.0):

16.4g	Sodium Acetate
1000ml	Double Distilled water

Visualisation Solution:

0.08g	Ammonium Chloride
2.5g	Nickel Ammonium Sulphate
100ml	0.1M Sodium Acetate
25mg	Diaminobenzidine tetrachloride (DAB; normally stored at 25mg/ml)

Slide Gelatinization Solution:

500ml	Distilled Water
2.5g	Gelatine
0.25g	Chromic Potassium Sulphate

Oxytocin Receptor Fixative (OTR; 1L):

1000ml	Glass Distilled Water
10.2g	Disodium hydrogen orthophosphate
3.85g	Sodium dihydrogen orthophosphate
40g	Paraformaldehyde
13.7g	L-Lysine
2.14g	Sodium m-periodate

Acid Alcohol:

700ml	Alcohol
300ml	Distilled Water
10ml	Hydrochloric Acid

Scott's Tap Water Substitute (STWS):

1000ml	Distilled Water
20g	Magnesium Sulphate
3.5g	Sodium Bicarbonate

1% Eosin:

100ml	Distilled Water
1g	Eosin

5% Potassium Aluminium:

100ml	Distilled Water
5g	Potassium Aluminium Sulphate

50mM Tris Buffer (pH 7.4):

50ml	1M Tris
1000ml	Distilled water

Tris $MgCL_2$ Buffer:

60.3g	Tris Base
20g	$MgCL_2$
500ml	Distilled water.

0.1% Paraformaldehyde in 50mM Tris Buffer:

1g	Paraformaldehyde
1000ml	50mM Tris Buffer

0.1% BSA in Tris $MgCL_2$ Buffer:

0.1g	BSA
100ml	Tris $MgCL_2$ Buffer

All reagents are below autoclaved prior to use to reduce/eliminate RNase activity.

0.1% DEPC- H_20:

1ml	Diethylpyrocarbonate
1000ml	Double Distilled water

4% Paraformaldehyde in Phosphate Buffer Saline (pH 7.2):

1000ml	DEPC- H_20
40g	Paraformaldehyde
8.0g	NaCl
0.2g	KCl
1.44g	NA_2HPO_4

TE Buffer (pH 7.4):

1000ml	Double Distilled water
10ml	1M Tris
2ml	0.5M EDTA

10X TBE Buffer:

1000ml	Double Distilled water
108g	Tris
55.7g	Boric Acid
4.7g	EDTA

20X Saline Sodium Citrate (SSC; pH 7.0):

1000ml	DEPC- H_20
175.3g	NaCl
88.2g	Tri-sodium Citrate

Deionised Formamide:

10g	Amberlite
100ml	‘AnalR’ Formamide

1M Dithiothretol (DTT):

0.82g	Dithiothretol
5ml	Sterile water

Appendix Six: Correlation graphs between Fos expression in specific brain regions and expression of specific maternal aggression components

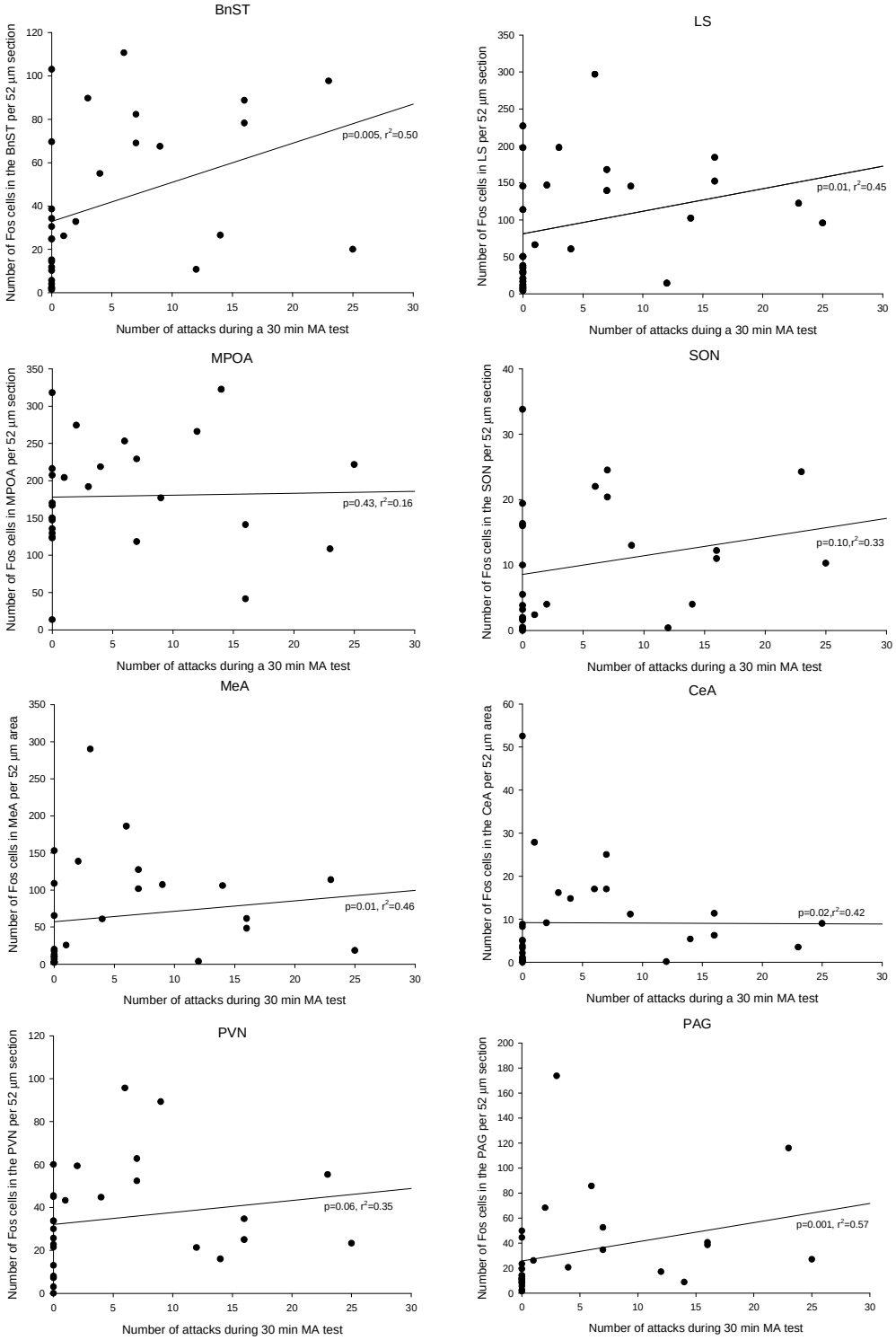


Figure 1: Correlations between number of attacks and Fos cells in specific brain regions of rats during a maternal aggression test. Graphs depicting Spearman correlations between the number of attacks and number of Fos positive cells in the lateral septum (LS), bed nucleus of stria terminalis (BnST), medial preoptic area (MPOA), supraoptic nucleus (SON), medial and central amygdala (MeA and CeA respectively), paraventricular nucleus (PVN) and periaqueductal grey (PAG) of lactating (n=30) and pup-sensitized (n=16) rats who have performed a 30 min maternal aggression test. Brains were collected for Fos immunocytochemistry from the female rats that were perfused 90 min after the start of the behavioural testing.

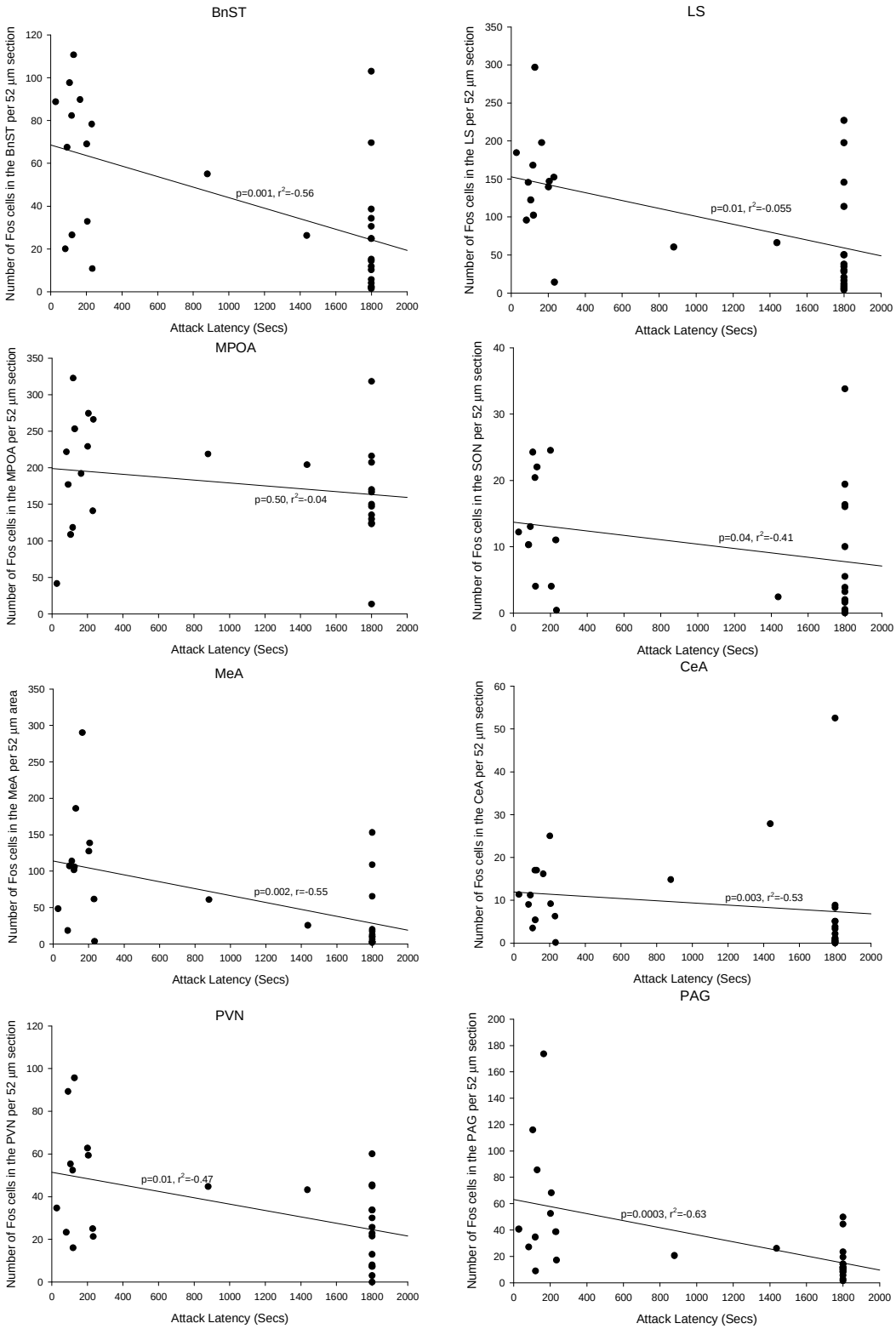


Figure 2: Correlations between attack latency and number of Fos cells in specific brain regions of rats during a maternal aggression test. Graphs depicting spearman correlations between the attack latency (secs) and number of Fos positive cells in the lateral septum (LS), bed nucleus of stria terminalis (BnST), medial preoptic area (MPOA), supraoptic nucleus (SON), medial and central amygdala (MeA and CeA respectively), paraventricular nucleus (PVN) and periaqueductal grey (PAG) of lactating (n=30) and pup-sensitized (n=16) rats who have performed a 30 min maternal aggression test. Brains were collected for Fos immunocytochemistry from the female rats that were perfused 90 min after the start of the behavioural testing.

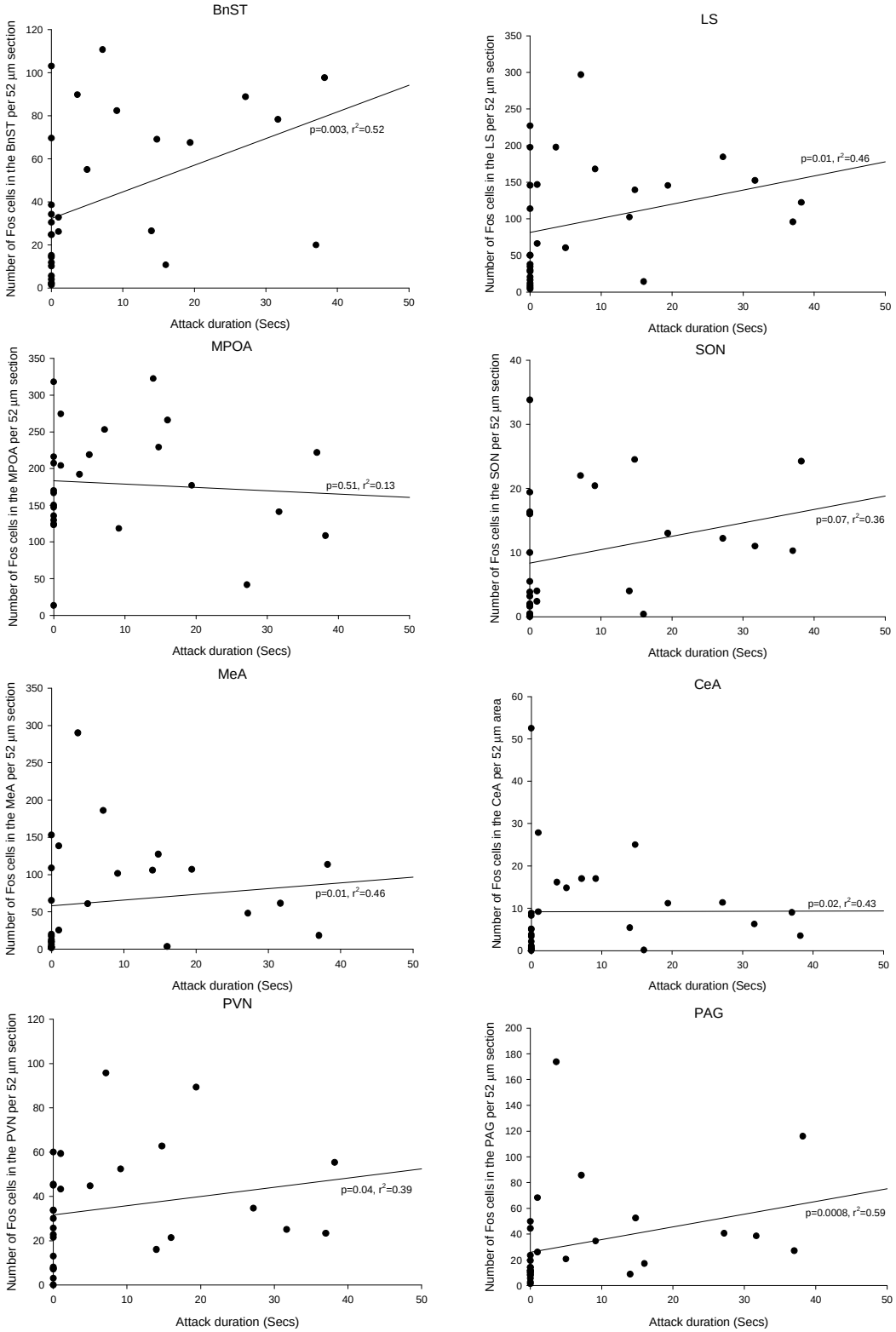


Figure 3: Correlations between attack duration and number of Fos cells in specific brain regions of rats during a maternal aggression test. Graphs depicting spearman correlations between attack duration (secs) and number of Fos positive cells in the lateral septum (LS), bed nucleus of stria terminalis (BnST), medial preoptic area (MPOA), supraoptic nucleus (SON), medial and central amygdala (MeA and CeA respectively), paraventricular nucleus (PVN) and periaqueductal grey (PAG) of lactating (n=30) and pup-sensitized (n=16) rats who have performed a 30 min maternal aggression test. Brains were collected for Fos immunocytochemistry from the female rats that were perfused 90 min after the start of the behavioural testing.

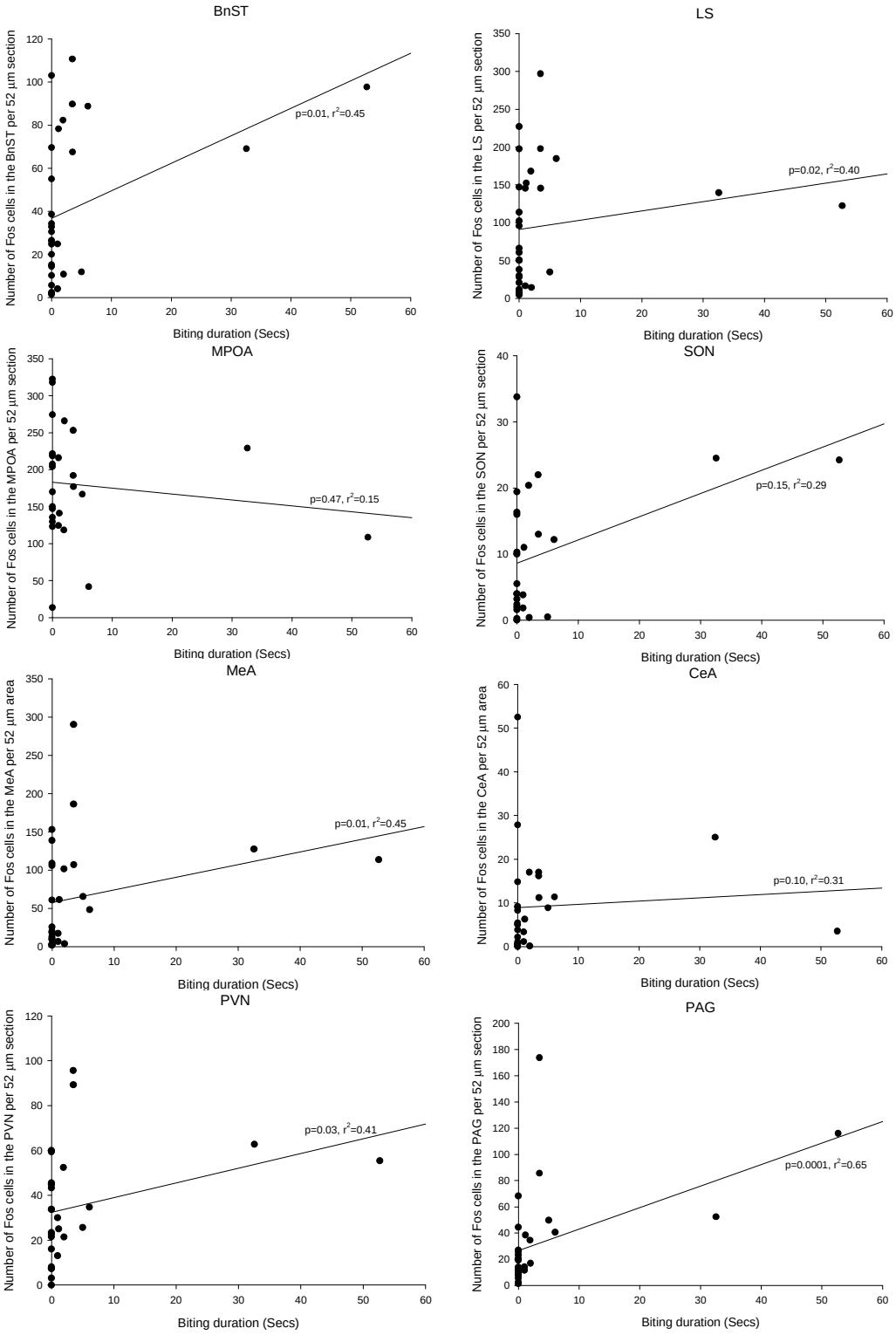


Figure 4: Correlations between biting duration and number of Fos cells in specific brain regions of rats during a maternal aggression test. Graphs depicting spearman correlations between the biting duration (secs) and number of Fos positive cells in the lateral septum (LS), bed nucleus of stria terminalis (BnST), medial preoptic area (MPOA), supraoptic nucleus (SON), medial and central amygdala (MeA and CeA respectively), paraventricular nucleus (PVN) and periaqueductal grey (PAG) of lactating (n=30) and pup-sensitized (n=16) rats who have performed a 30 min maternal aggression test. Brains were collected for Fos immunocytochemistry from the female rats that were perfused 90 min after the start of the behavioural testing.

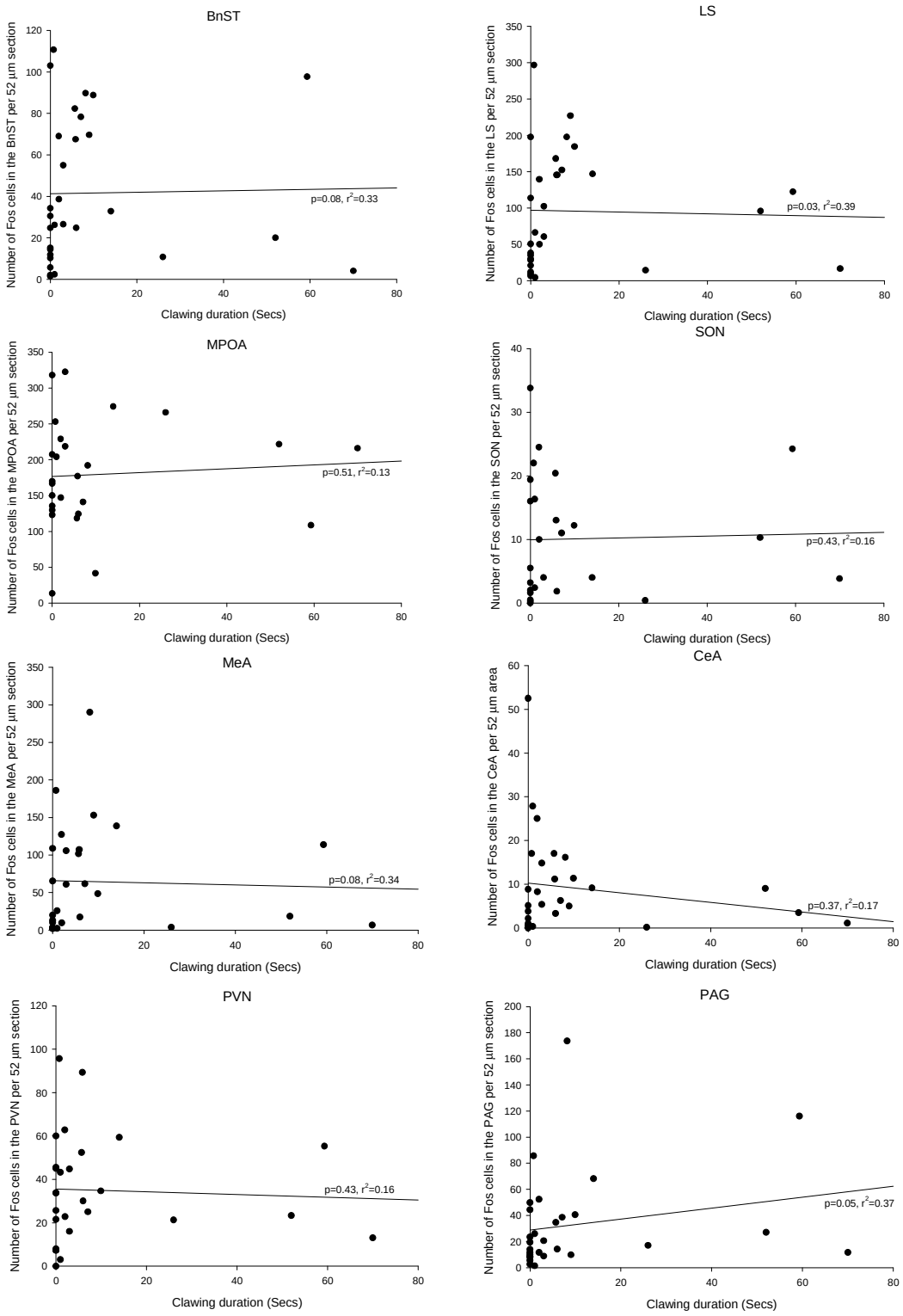


Figure 5: Correlations between clawing duration and number of Fos cells in specific brain regions of rats during a maternal aggression test. Graphs depicting spearman correlations between clawing duration (secs) and number of Fos positive cells in the lateral septum (LS), bed nucleus of stria terminalis (BnST), medial preoptic area (MPOA), supraoptic nucleus (SON), medial and central amygdala (MeA and CeA respectively), paraventricular nucleus (PVN) and periaqueductal grey (PAG) of lactating (n=30) and pup-sensitized (n=16) rats who have performed a 30 min maternal aggression test. Brains were collected for Fos immunocytochemistry from the female rats that were perfused 90 min after the start of the behavioural testing.

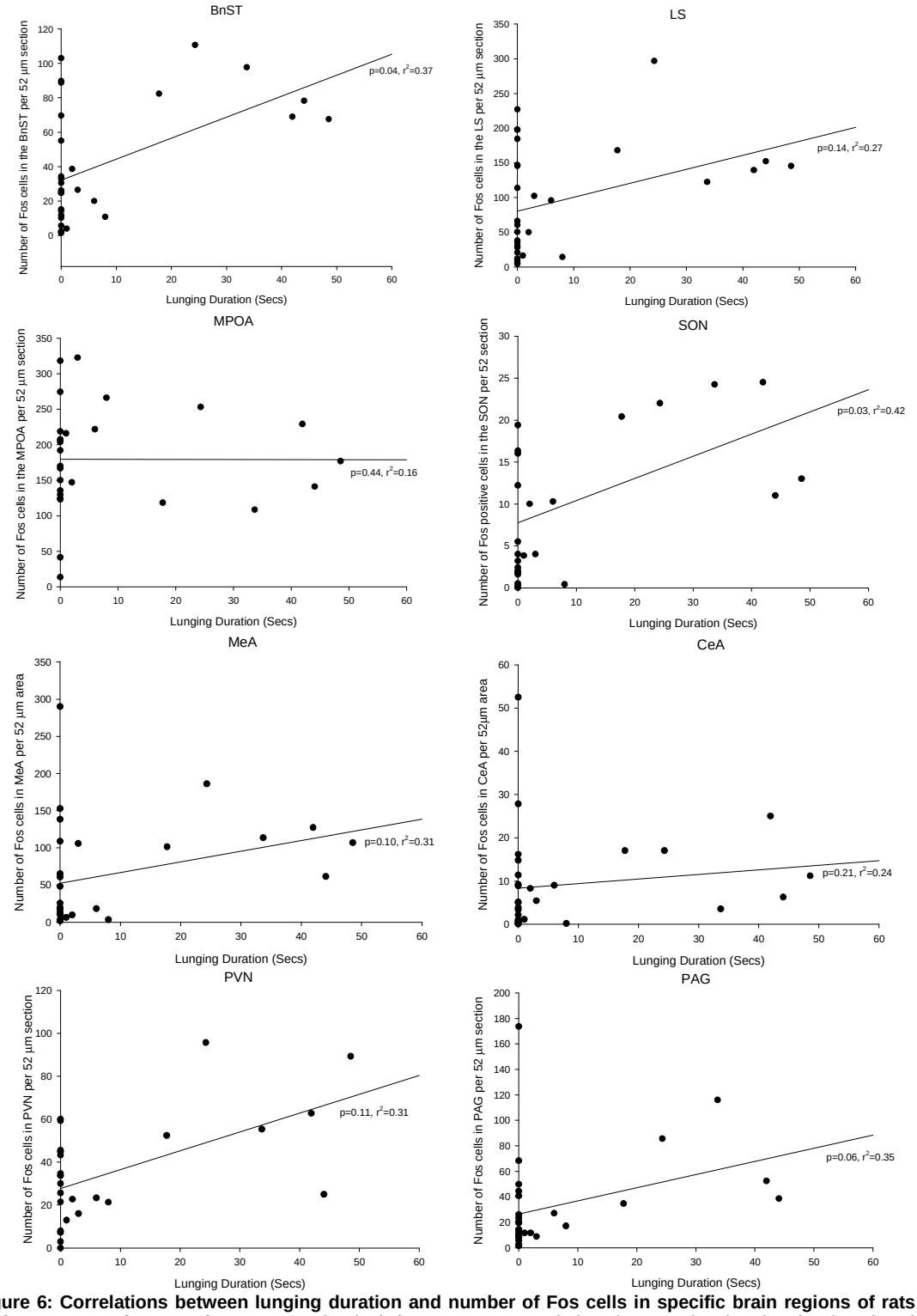


Figure 6: Correlations between lunging duration and number of Fos cells in specific brain regions of rats during a maternal aggression test. Graphs depicting spearman correlations between lunging duration (secs) and number of Fos positive cells in the lateral septum (LS), bed nucleus of stria terminalis (BnST), medial preoptic area (MPOA), supraoptic nucleus (SON), medial and central amygdala (MeA and CeA respectively), paraventricular nucleus (PVN) and periaqueductal grey (PAG) of lactating (n=30) and pup-sensitized (n=16) rats who have performed a 30 min maternal aggression test. Brains were collected for Fos immunocytochemistry from the female rats that were perfused 90 min after the start of the behavioural testing.

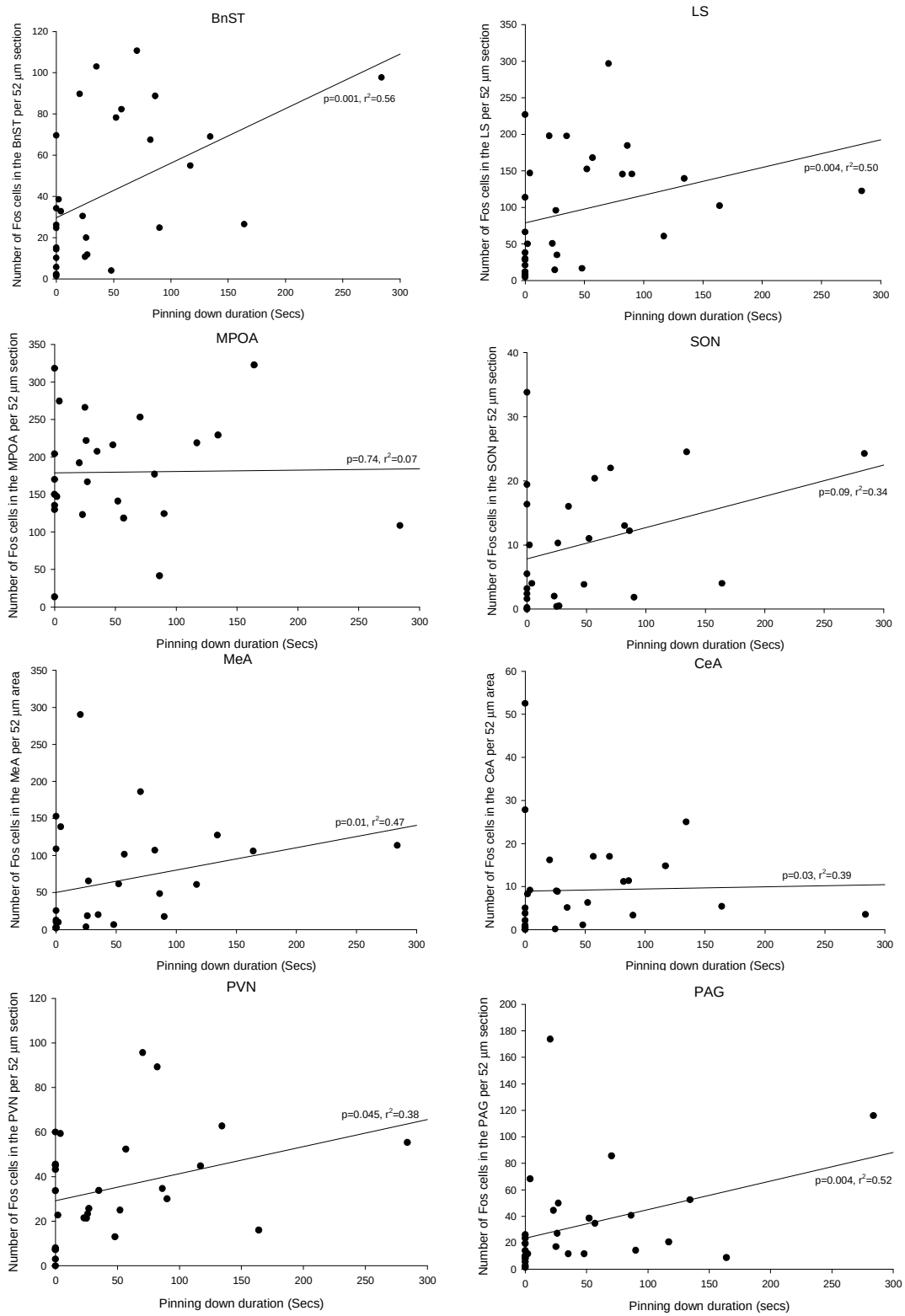


Figure 7: Correlations between pinning down duration and number of Fos cells in specific brain regions of rats during a maternal aggression test. Graphs depicting spearman correlations between pinning down duration (secs) and number of Fos positive cells in the lateral septum (LS), bed nucleus of stria terminalis (BnST), medial preoptic area (MPOA), supraoptic nucleus (SON), medial and central amygdala (MeA and CeA respectively), paraventricular nucleus (PVN) and periaqueductal grey (PAG) of lactating (n=30) and pup-sensitized (n=16) rats who have performed a 30 min maternal aggression test. Brains were collected for Fos immunocytochemistry from the female rats that were perfused 90 min after the start of the behavioural testing.

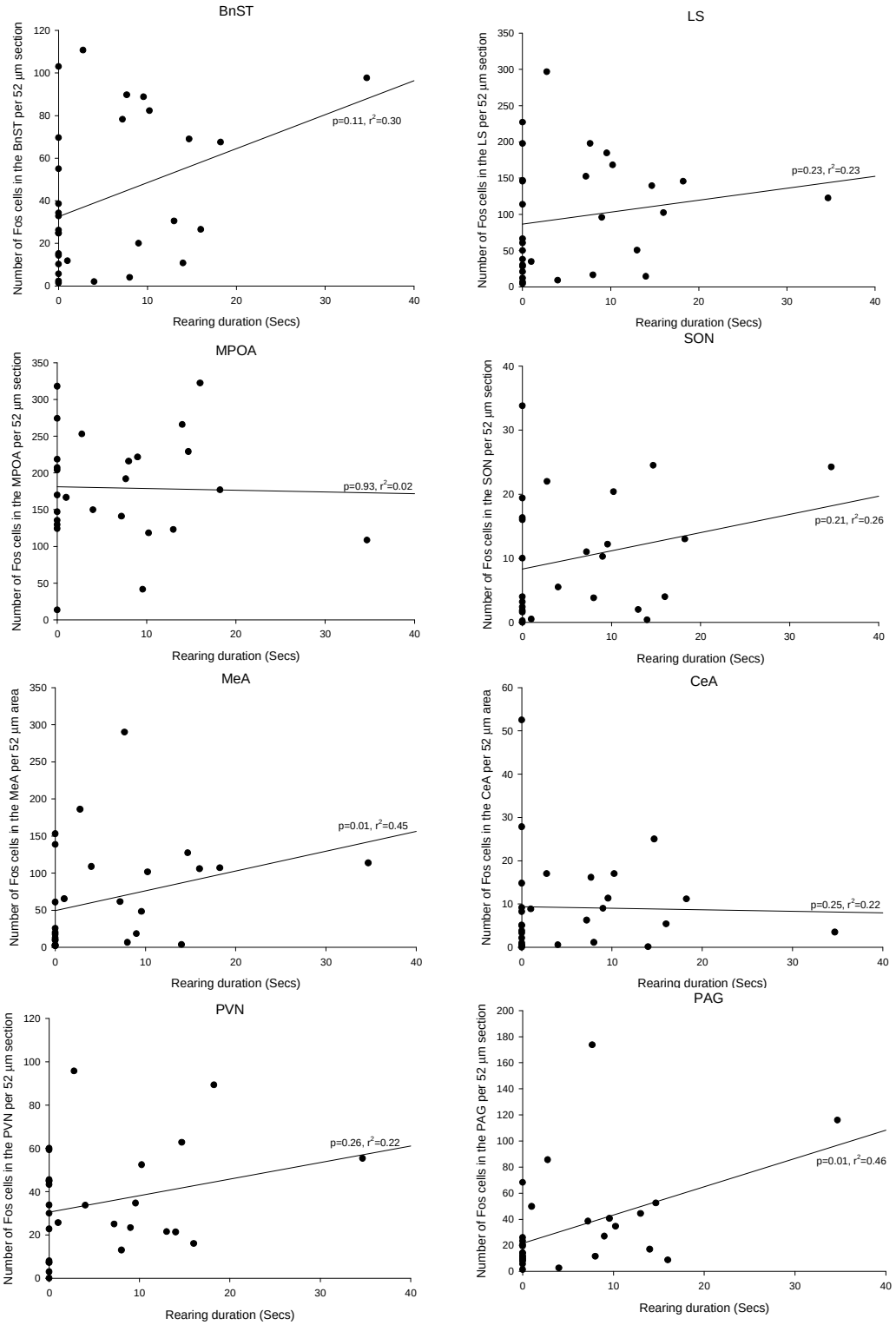


Figure 8: Correlations between rearing duration and number of Fos cells in specific brain regions of rats during a maternal aggression test. Graphs depicting spearman correlations between rearing duration (secs) and number of Fos positive cells in the lateral septum (LS), bed nucleus of stria terminalis (BnST), medial preoptic area (MPOA), supraoptic nucleus (SON), medial and central amygdala (MeA and CeA respectively), paraventricular nucleus (PVN) and periaqueductal grey (PAG) of lactating (n=30) and pup-sensitized (n=16) rats who have performed a 30 min maternal aggression test. Brains were collected for Fos immunocytochemistry from the female rats that were perfused 90 min after the start of the behavioural testing.

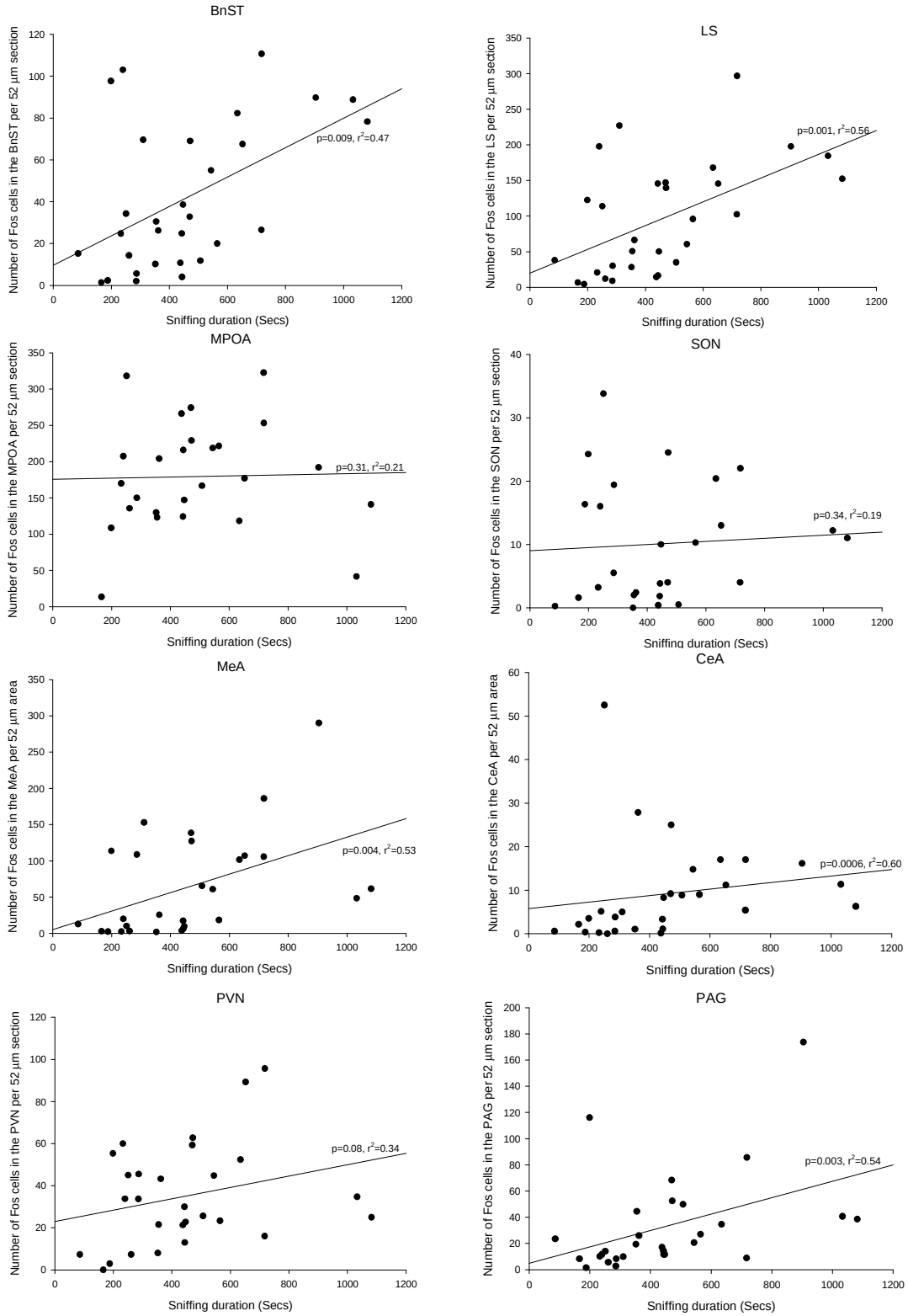


Figure 9: Correlations between sniffing duration and number of Fos cells in specific brain regions of rats during a maternal aggression test. Graphs depicting spearman correlations between sniffing duration (secs) and number of Fos positive cells in the lateral septum (LS), bed nucleus of stria terminalis (BnST), medial preoptic area (MPOA), supraoptic nucleus (SON), medial and central amygdala (MeA and CeA respectively), paraventricular nucleus (PVN) and periaqueductal grey (PAG) of lactating (n=30) and pup-sensitized (n=16) rats who have performed a 30 min maternal aggression test. Brains were collected for Fos immunocytochemistry from the female rats that were perfused 90 min after the start of the behavioural testing.

Appendix Seven: Chemical index

Reagent	Company	Code
Ab-2 Fos antibody	Calbiochem	PC05-100µg
Acetic anhydride	Sigma	A6404
Allopregnanolone	Steraloids	P3800-000
Amberlite	BDH	551794K
Ammonium chloride	Sigma	A4514
'AnalR' formamide	BDH	103264R
ATP, CTP, GTP	Promega	
Bicuculline Methiodide	Sigma	14343
Biotinylated anti rabbit Ig	Vector	BA-1000
Boric acid	Sigma	B7660
Bovine serum albumin	Sigma	
Chloroform:Isoamyl alcohol	Sigma	C0549-1PT
Chromium potassium sulphate 12 hydrate	BDH	2775845
Deionised Formamide	Sigma	F9037
Developer Kodak D19	Ilford/Lizars	
Diaminobenzidine tetrachloride (DAB)	Sigma	D5637
Diethylpyrocarbonate (DEPC)	Sigma	D57580
DPX mountant	VWR	360294
Disodium hydrogen orthophosphate	BDH	103834G
Dithiothretol (DTT)	Sigma	D9779
Emulsion (Ilford)	Calumet	P9281
Ethylene diamine tetracetic acid (EDTA)	Sigma	E7889
Ethylene glycol	Sigma	102466
Finasteride	Steraloids	P3500-000
GAD 65/67 antibody	Chemicon	AB1511
Gelatine	BDH	440454B
Glycerol	Fisher Scientific	G/0600/17
Heparin	Sigma	H0777-500KU
Hydrogen peroxide (30%)	Sigma	H1009
L-Lysine	Sigma	L5501
Magnesium chloride	Sigma	M8266
Magnesium sulphate	Sigma	M7506
Methyl green	Sigma	M8884
Nickel ammonium sulphate	Fisher Scientific	S/3160/63
NICK column (Sephadex)	Amersham	17-0855-01
Normal sheep serum	Sigma	S2263
Oxytocin receptor	Perkin Elmer	NEX2540 10µCi
Paraformaldehyde	Sigma	P6148

Phenol:Chloroform isoamyl alcohol	Sigma	P3803
Potassium chloride	Sigma	P3911 or 9541
Progesterone radioimmunoassay	Oxford Bioinnovations	DSL 3900
Salmon sperm DNA	Sigma	D9156
Simplex rapid liquid	Kemdent	ACR921
Simplex rapid powder	Kemdent	ACR806
Sodium acetate	Sigma	S9513
Sodium bicarbonate	Sigma	S8875
Sodium chloride	Fisher Scientific	A/5000/53
Sodium dihydrogen orthophosphate	BDH	1024555
Sodium m-periodate	Sigma	S1878
Sucrose	Sigma	S7903 or 179949
SYBR safe DNA gel stain	Invitrogen	533102
Toludine blue	Sigma	
Triethanolamine	Sigma	T1377
Tris	Sigma	T8524
Tri-sodium citrate	Sigma	S4641
Triton X-100	Sigma	T9284
Vasopressin receptor	Perkin Elmer	NEX3100 10μCi
Vectastain ABC elite kit	Vector Labs	PK6100
Xylene	BDH	1330-20-7

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